UNIVERSITY OF LJUBLJANA
BIOTECHNICAL FACULTY

Sandra HOČEVAR

ISOLATION AND CHARACTERIZATION OF PURE GLIAL CELL POPULATIONS FROM ADULT MOUSE CENTRAL NERVOUS SYSTEM WHITE MATTER

M.Sc. Thesis

Ljubljana, 2014

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IZOLACIJA IN KARAKTERIZACIJA ČISTIH GLIJA CELIČNIH POPULACIJ IZ BELINE CENTRALNEGA ŽIVČNEGA SISTEMA ODRASLE MIŠI

Magistrsko delo

Ljubljana, 2014

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AB Glial cells are critical participants in the development, function and disease of the central nervous system (CNS). They play roles in brain homeostasis, synapse formation, myelin production, and provide the immune system for CNS. There are several *in vitro* glia studies which mostly include experiments on embryonic or young postnatal rodent brains. Therefore, it is important to find a way to isolate pure glia from adult mouse CNS, since all the major nerurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD) and Multiple sclerosis (MS), occur in adulthood. With this aim, we tried to develop a reliable technique for isolating purified astrocytes and oligodendrocytes from adult mouse brain and optic nerve. Our experiments were based on two different methods of cell dissociation and isolation, using the Magnetic Activated Cell Sorter (MACS) system. We managed to dissociate, culture and characterise populations of oligodendrocytes, astrocytes and oligodendrocyte precursor cells (OPCs) (or oligodendrocyte-type 2 astrocyte progenitors-O-2A) from adult mouse CNS. Our optimised in-house dissociation method proved as useful and equally successful as the Neural Tissue Dissociation Kit (NTDK) method. However, cell isolation from adult tissue with MACS system turned out to be impracticable and remains difficult to perform.

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AI Celice glija so pomembne udeleženke v razvoju, funkciji in boleznih centralnega živčnega sistema (CŽS). Igrajo vloge pri homeostazi v možganih, produkciji mielina in zagotavljajo imunski sistem CŽS. Večina študij glije *in vitro* vključuje poskuse na embrionalnih ali mladih postnatalnih možganih glodalcev. Ker pa se večina poglavitnih nevrodegenerativnih bolezni, kot so Alzheimerjeva bolezen, Parkinsonova bolezen in multipla skleroza, pojavljajo pri odraslem človeku, je pomembno, da najdemo način za izolacijo čiste glije iz CŽS odrasle miši. S tem namenom smo poskušali razviti zanesljivo tehniko izolacije čistih astrocitov in oligodendrocitov iz možganov in optičnega živca odrasle miši. Naši poskusi so temeljili na dveh različnih disociacijskih metodah in izolaciji z magnetno pogojenim ločevanjem celic (*ang*. MACS). Iz CŽS odrasle miši nam je uspelo disociirati, kultivirati in karakterizirati populacije oligodendrocitov, astrocitov in prekurzorskih celic oligodendrocitov (*ang*. OPCs) (ali oligodendrocit-astrocit tipa 2- O-2A). Dokazali smo, da je naša optimizirana disociacijska metoda enako uspešna kot disociacijska metoda z uporabo kompleta za disociacijo živčnega tkiva (*ang*. NTDK). Vendar pa se je izolacija celic s sistemom MACS iz odraslega tkiva izkazala za težavno in se je ne uporablja.

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ABBRIVATIONS AND SYMBOLS

AD	Alzheimer's disease
APC	Adenomatous polyposis coli
Aβ	Aβ-amyloid protein
BBB	Blood-brain barrier
C1q	Complement component 1 q
CK1	Casein kinase 1
CNS/*CŽS*	Central nervous system/*centralni živčni sistem*
Dvl	Dishevelled
EAAT1	Excitatory amino acid transporter 1
eGFP	Enhanced green fluorescent protein
GABA	Gamma-aminobutyric acid
GFAP	Glial fibrilary acidic protein
GFP	Green fluorescent protein
GLAST	Glutamate aspartate transporter
Gro	Groucho
GSK-3β	Glycogen synthase kinase-3β
HDAC	Histone deacetylases
HMG	High mobility group
IF	Intermediate filament
IP_3	Inositol trisphosphate
IPCs	Intermediate progenitor cells
LEF	Lymphoid enhancer factor
LRP5/6	LDL receptor-related proteins 5 and 6
MACS	Magnetic activated cell sorter
MAG	Myelin associated glycoprotein
MBP	Myelin basic protein
MS	Multiple sclerosis
NSCs	Neural stem cells
NG2	Neuron glia 2
NTDK	Neural tissue dissociation kit

Olig1	Oligodendrocyte transcription factor 1
Olig2	Oligodendrocyte transcription factor 2
OPCs	Oligodendrocyte progenitor cells
PD	Parkinson’s disease
PDGF	Platelet-derived growth factor
PDGFαR	Platelet-derived growth factor α receptor
PLP	Proteolipid protein
PNS/*PŽS*	Peripheral nervous system/*periferni živčni sistem*
RT	Room temperature
SVZ	Subventricular zone
TCF	T-cell factor
TLE	Transducin-like enhancer
TNFα	Tumor necrosis factor α
qPCR	Quantitative polymerase chain reaction

1 INTRUDUCTION

Our central nervous system (CNS) consists of neurons and glial cells. The defining characteristic of a neuron is its ability to transmit rapid electrical signals in the form of action potentials, whereas glial cells are unable to generate action potentials but instead surround and ensheath neuronal cell bodies, axons and synapses throughout the nervous system (Allen and Barres, 2009). Neurons and glia have a common origin in neuronal precursor cells derived from the embryonic germ layer known as the neuroectoderm. Astrocytes, oligodendrocytes and ependymal cells are cells of neural origin and often referred to as macroglial or neuroglial cells. The remaining 5-10 % of glia consist of microglia, which are a part of immune system and have non-neuronal (mesodermal) origin. In the peripheral nervous system (PNS), the main class of glia is represented by Schwann cells which enwrap and myelinate peripheral axons (Verkhratsky and Butt, 2007).

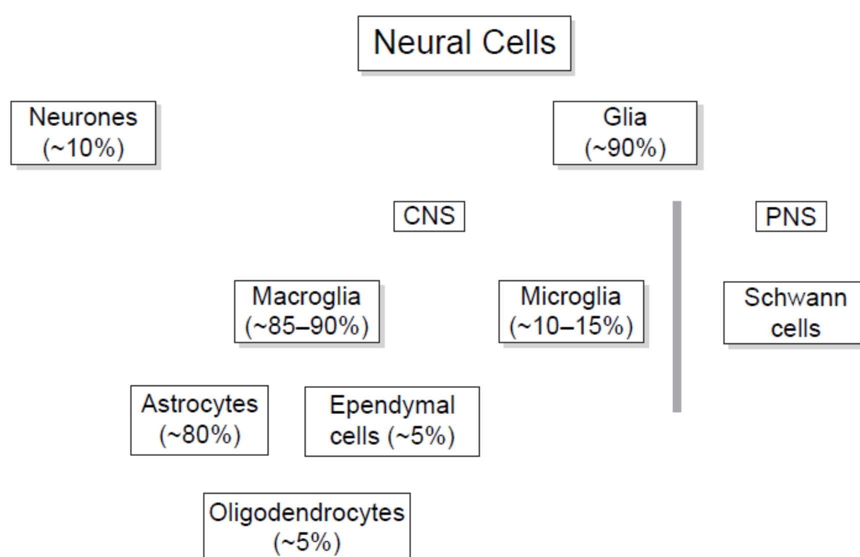

Figure 1: Neural cell types. CNS: Central nervous system, PNS: peripheral nervous system (Verkhratsky and Butt, 2007: 4)

Glial cells play multiple roles in adult neurogenesis, ranging from functioning as neural precursor cells to the key determinants of neurogenic permissiveness (Morrens et al., 2012). Astrocytes are fundamental for brain homeostasis, are critical at the brain-blood

barrier (Heneka et al., 2009) and provide structural and metabolic support to the nervous system (Haydon and Carmignoto, 2006).

The human CNS is the most complex system of cells known. Much of our understanding of normal human brain functions and pathological processes involved in neurodegeneration have come through the use of *in vivo* and *in vitro* non-human models. Studies using primary cultures and cell lines of neurons, microglia, astrocytes and oligodendrocytes are numerous and are used to investigate many processes, such as neurotoxicity, inflammation and neuroprotection, as well as being used to develop novel drugs to treat neurodegenerative disorders (Allen et al., 2005; Petrova et al., 2004). They are also useful tools when studying cell differentiation, proliferation, physiology and morphology in a controlled environment with maintenance of *in vivo* cell characteristics (Megale de Almeida-Leite and Esteves Arantes, 2010). In addition, glial cells play a great part in neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and Multiple sclerosis (MS) (Heneka et al., 2010). However, most *in vitro* studies use cells derived from embryonic and postnatal brains, whereas the majority of neurodegenerative diseases occur in adult brains. It is therefore important to develop models that allow us to study adult glia *in vitro* so as to develop potential therapeutics on adult humans.

1.1 AIM OF STUDY

Our aim was to develop a reliable technique for isolating purified astrocytes and oligodendrocytes from adult mouse CNS using model white matter tissue of the optic nerve and brain.

1.2 HYPOTHESIS

Astrocytes and oligodendrocytes from adult mouse CNS can be isolated by using the Magnetic Activated Cell Sorter (MACS) system, and maintained in culture for cell biological experiments.

2 LITERATURE REVIEW

2.1 BACKGROUND OF GLIA

The concept and term of glia was introduced for the first time in 1858 by Rudolf Virchow. He derived the term glia (γλια) from the Greek word for something slimy and sticky in appearance. Camillo Golgi discovered groups of cells located between nerve fibres during his first observation of oligodendrocytes. The term astrocyte came from the Greek word for star (astro) and cell (cyte) and was proposed by Michael von Lenhossek in 1893 to describe stallete shaped glia (Verkhratsky and Butt, 2007). Del Rio-Hortega introduced the term 'microglia' to describe a new central nerve cell type that he considered to be derived from mesodermal elements (Somjen, 1988) and understood that these cells have the ability to migrate and act as phagocytes. In 1894, Carl Ludwig Schleich was the first to claim that neurons and glia have equally important roles and are active cellular elements in the human brain (Verkhratsky and Butt, 2007). Once glial cells had been properly differentiated from neurons, the door was opened to speculation over their functions (Somjen, 1988).

To this day the main types of glia remained the same: astrocytes, oligodendrocytes and microglia. However, in the recent decade, another class of glia was identified; NG2 positive glia, otherwise considered as oligodendrocyte progenitor cells (OPCs) (Butt et al., 2005). The cellular elements comprising each of these groups, although having common features, demonstrate a profound functional heterogeneity in different brain regions and at different developmental stages (Heneka et al., 2010). Moreover, glia became far more important in health and disease then once thought (Barres, 2008).

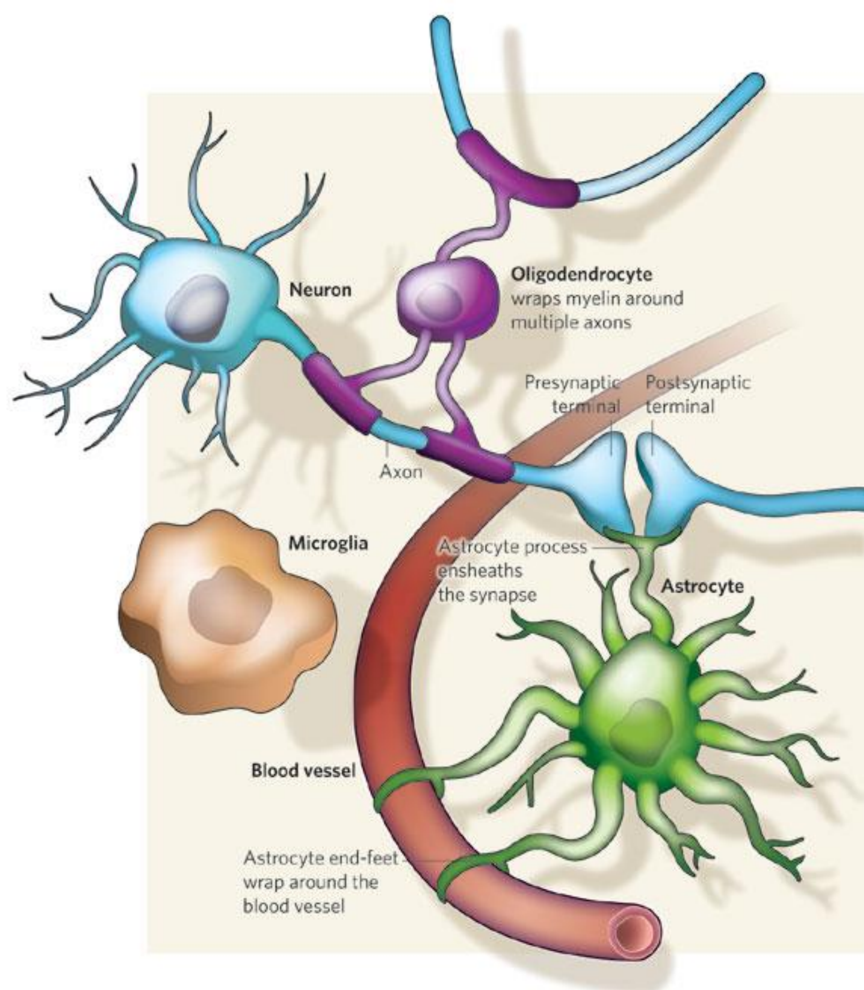

Figure 2: Glia-neuron interactions (Allen and Barres, 2009: 675)

2.2 ASTROCYTES

Astrocytes are the most numerous cells in the mammalian brain. These cells develop from astrocyte precursors, called radial glia, which participate in neuronal migration during the embryonic brain development (Anton et al., 1996; Gomes et al., 1999). In the postnatal brain, astrocytes become a part of heterogeneous family of morphologically distinct cells and have several important functions in the brain (Gomes et al., 1999; Molofsly et al., 2012).

2.2.1 Astrocyte morphology and functions

Morphologically we distinguish two types of astrocytes. Fibrous (type 1 *in vitro*) astrocytes, which populate the white matter and typically have regular contours and

cylindrical processes, yielding the more classic 'star like' appearance, with dense glial filaments that stain with the intermediate filament marker, glial fibrilary acidic protein (GFAP) (Fig. 3A) (Vaughn and Pease, 1967, cit. by Molofsly et al., 2012). This type of astrocytes contact nodes of Ranvier and vessels in white matter (Barres, 2008). Protoplasmic (type 2 *in vitro*) astrocytes populate the grey matter and have more irregular processes and few glial filaments. This type of astrocytes contact and ensheath synapses by extending thousands of thin processes, with typically only one or two in contact with blood vessels or CNS boundaries (Fig. 3B,C) (Bushong et al., 2002).

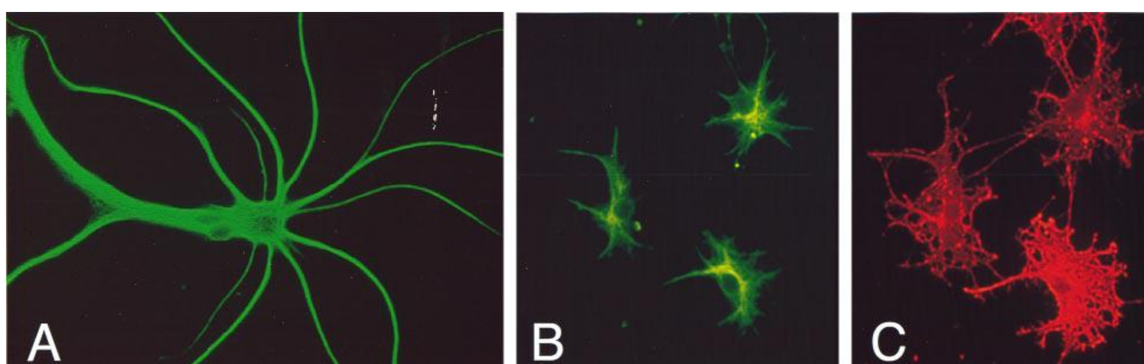

Figure 3: Astrocyte subtypes: fibrous (type 1 *in vitro*) astrocyte (A) and protoplasmic (type 2 *in vitro*) astrocytes (B, C); cells are marked with GFAP marker (A, B) and A2B5 marker (C) (Compston et al., 1997: 166)

The main function of astrocytes is to allow neurons to function. Firstly, astrocytes are responsible for the regulation of the microenvironment. They remove excess neurotransmitters and ions from extracellular space (Heneka et al., 2010). For example, the accumulation of extracellular potassium is particularly important as it accompanies the repolarisation phase of action potentials. The control of the extracellular K^+ concentrations is driven by astrocytes trough local K^+ uptake involving inward rectifier K^+ channels and K^+ spatial buffering (Kofuji and Nevman, 2004). Furthermore, astrocytes control glutamate concentrations in the extracellular space. From the bulk of glutamate released during synaptic transmission, about 20 % is accumulated into postsynaptic neurones and the remaining 80% is taken up by perisynaptic astrocytes (Verkhratsky and Butt, 2007).

Astroglia enable metabolic support to neurons by collection of neuronal waste and delivery of nutrients to neurons (Verkhratsky and Butt, 2007). These cells accumulate about 50 %

of glucose entering the brain tissue, and store it in the form of glycogen. Glycolysis is stimulated by increase of Na^+ concentration trough Na^+- dependent glutamate transport into the astrocytes. This results in synthesis of lactate, which is transported to the neurons and provides them with energy (Magistretti, 2009).

Astrocytes are linked together by the structures in their cell bodies called gap junctions, which represent an information-transfer system and allow communication between each other by diffusion of a second messenger like inositol trisphosphate (IP_3) and Ca^{2+} release. Ca^{2+} propagates the so-called calcium waves between astrocytes, which allow transfer of information even over long distances (Dani et al., 1992; Scemes and Giaume, 2006).

Astrocytes form neuronal-glial-vascular units, which integrate with neural circuitry with local blood flow (Heneka et al., 2010). They also build a functional link between neurones and blood vessels. Astrocyte Ca^{2+} waves can trigger the release of vasoactive substances, which results in either vasoconstriction or vasodilatation (Metea and Newman, 2006). In the adult brain, astrocytic endfeet form a sheathing network around the brain vasculature known as the glia limitans, which, together with pericytes and endothelial cells, form a barrier to the passage of molecules, ions, and cells from the bloodstream into the brain parenchyma: the blood-brain barrier (BBB) (Molofsky et al., 2012). This represents an important role of astrocytes in the regulation of cerebral blood flow (Takano et al., 2006) and BBB permeability (Zlokovic, 2008).

The ability of astrocytes to release neuroactive substance (gliotransmitters) and act on synapse is called gliotransmission (Heneka et al., 2010). This process is induced by Ca^{2+} waves in astrocytes, which induce the secretion of gliotransmitters such as glutamate, ATP, D-serine, GABA, taurine (Kozlov et al., 2006; Oliet and Mothet, 2009). This has led to the concept of the tripartite synapse - the astroglial perisynaptic process, presynaptic neuronal terminal and postsynaptic neuronal membrane (Araque et al., 1999). The role of astrocytes in this synapse is dual. First, they sense the transmitter released from the neuronal terminal by the numerous receptors that are expressed on the astroglial membrane, and secondly, by releasing gliotransmitters, the astrocyte can control the efficacy of the synapse (Heneka et al., 2010). In addition, astrocytes also provide the micro-architecture of the brain by

forming a scaffold to the nervous system (Verkhratsky and Butt, 2007), have control of water homeostasis (Simard and Nedergaard, 2004) and may also help oligodendrocytes in myelination of axons (Meyer-Franke et al., 1999, cit. by Molofsky et al., 2012).

2.2.2 Astrocyte markers

Astroglia express astrocyte specific antigens, which represent important tools for their detection and characterisation.

Table 1: Astrocyte markers

Marker	Function
GLAST/EAAT1	Glutamate Aspartate Transporter (GLAST) or Excitatory Amino Acid Transporter 1 (EAAT1) is a Na^+-dependent high-affinity glutamate transporter (Sheldon and Robinson, 2007).This type of glutamate transporters are predicted to have 8 transmembrane domains and most likely exist as trimmers (Yernool et al., 2003). GLAST was primarily found in the plasma membranes of astrocytes (Rothstein, 1994) and in radial glia-astrocyte lineage of developing rodent CNS (Shibata et al., 1997). However, it could also be found in many other cells, such as oligodendroglia (Domercq, et al., 1999) and macrophages (Gras et al., 2003). GLAST is enriched in astrocytic processes near synaptic termini (Chaudhry et al., 1995) and increased in some specialized parts of the brain such as Bergmann glia and Muller cells of the retina (Sheldon and Robinson, 2007). Its main function is to clear out extracellular glutamate throughout the synaptic cleft, to keep the level low enough to prevent neurons from excitotoxicity. Furthermore, GLAST is involved not only in the regulation of synaptic transmission, but it also plays some roles in the development and differentiation of the CNS (Shibata et al., 1997).
GFAP	GFAP comes from a large multigene family of intermediate filament (IF) proteins. It is sorted into Class III of IF family, which represents astroglial characteristic. GFAP is a major IF protein of mature astrocytes and is known as maturation marker (Gomes et al., 1999). GFAP was found to be important for integrity of CNS white matter architecture and long-term maintenance of myelination. It also plays an important role in the formation of BBB (Liedtke, et al., 1996).
Vimentin	Vimentin is a Class III IF protein and is present in all mesenchymal tissues. It is expressed in a wide variety of cells including astrocytes, fibroblasts, endothelial cells, macrophages, neutrophils and lymphocytes (Evans, 1998). Vimentin is the major IF expressed in astrocyte precursors and radial glia. During the maturation of astrocyte, its expression is progressively replaced by expression of GFAP (Gomes et al., 1999). Vimentin's major function is to build cytoskeletal structures deep inside the cell and it plays an important role in supporting the location of the organelles (Katsumoto et al., 1990).

2.2.3 Astrocyte pathology

2.2.3.1 Reactive astrogliosis

Because astrocytes represent almost one half of the brain cells, there is practically no CNS disease that does not involve their participation. Astrocytes are important defence

mechanisms and are genuinely responsible for the protection and survival of the neural tissue. The reactive astrogliosis is essential for both limiting the areas of damage and for the post-insult remodelling and recovery of neural function (Henaka et al., 2010). However, this mechanism can also act as a harmful factor by trying to seal and eliminate the damage (Barres, 2008). In addition, reactive astrocytes can stimulate unwanted synapses that can cause epilepsy, neuropathic pain and are involved in several other neuro-pathological conditions (Boroujerdi et al., 2008).

2.2.3.2 Alzheimer's disease

Astrocytes are involved in a variety of psychiatric disorders, e.g. depression and schizophrenia, and neurodegenerative diseases, e.g. Parkinson's disease (PD), Alzheimer's disease (AD) and other types of dementia (Heneka, et al., 2010). AD is the main cause for senile dementia. The results of this disease are rapid memory lost and severe disruption of cognitive functions. The main pathological markers of AD are the formation of deposits of β-amyloid protein (Aβ) in the walls of blood vessels, accumulation of Aβ plaques in the grey matter and intra-neuronal accumulation of abnormal tau-protein filaments in the form of neurofibrillary tangles (Verkhratsky and Butt, 2007). Aβ was found to be an activating signal for astrocytes. The exposure of cultured glial cells to aggregated Aβ or amyloid plaques triggered harmful reactive astrogliosis (DeWitt, et al., 1997). Thus, it is possible to observe astrogliosis in adult AD brain, especially in the cells surrounding amyloid plaques with processes of activated astrocytes joining in formation of neurotic plaques (Nagele et al., 2004).

2.2.3.3 Parkinson's disease

PD is a neurodegenerative disorder, theclinical symptoms of which are disturbed locomotive and motor functions (Parkinson, 2002). These symptoms occur because of the specific extermination of dopaminergic neurons in substantia nigra, which has severe effect on nigrostriatal dopaminergic transmission. At the late stages of the disease, reactive astrocytes are involved in the inflammatory state (McGeer and McGeer, 2008) and may also fail to support dopaminergic neurons, which contributes to neurodegeneration (Mena,

et al., 2002). However, it is not fully known what the early changes in astroglia are, although they may be responsible for the progression of the disease (Heneka et al., 2010).

2.3 OLIGODENDROCYTES

Oligodendrocytes are myelinating cells in the CNS. They develop from migratory and mitotic OPCs (Baumann and Pham-Dinh, 2001). From their germinal zones, OPCs migrate to their target regions in white and grey matter, where they maturate into mature myelin-producing cells and begin to wrap axons by forming myelin sheaths (Fig. 4) (McTigue and Tripathi, 2008).

2.3.1 Oligodendrocyte morphology and myelin functions

Oligodendrocytes can be distinguished from astrocytes by several features, especially their smaller size, higher density of cytoplasm and nucleus with dense chromatin, the absence of intermediate filaments and glycogen in cytoplasm, and the presence of a large number of microtubules in their processes that might be involved in their stability (Baumann and Pham-Dinh, 2001; Lunn et al., 1997). On the same axon, myelin sheaths belong to different oligodendrocytes. Moreover, these cells are able to enwrap up to 50 axonal segments, depending on the region of the CNS (Baumann and Pham-Dinh, 2001). By their morphology, size or thickness of the myelin sheath they form, we distinguish four different types of oligodendrocytes. Oligodendrocyte types I and II arise late in the development and myelinate many internodes on small diameter axons, while oligodendrocyte types III and IV arise later and myelinate mainly large diameter axons (Butt, 2013). Though oligodendrocytes are mostly myelin producing cells, there also exist satellite oligodendrocytes that may not be directly involved in the formation of myelin sheath. The satellite oligodendrocytes are perineuronal and most likely serve to regulate the microenvironment around neurons (Ludwin, 1997, cit. by Baumann and Pham-Dinh, 2001).

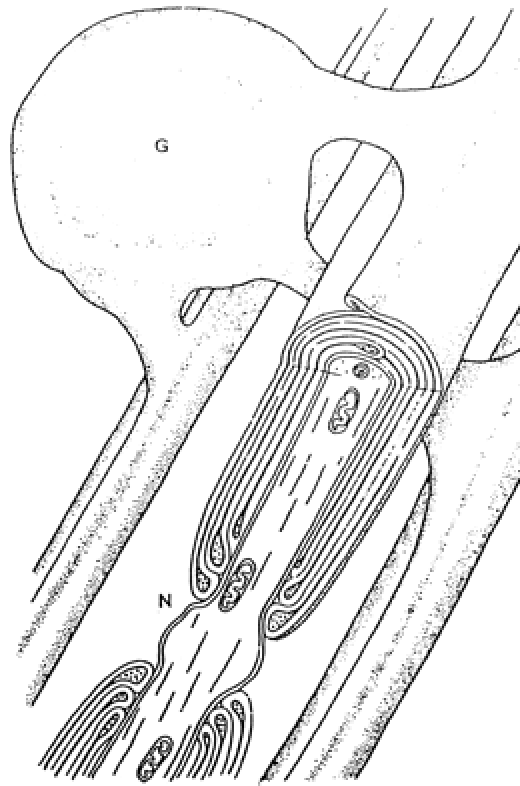

Figure 4: The oligodendrocyte and the mature myelin sheath. Oligodendrocyte- G, node- N (Baumann and Pham-Dinh, 2001: 873)

The main and most evident function of oligodendrocytes is the production of myelin, which is significant for the isolation of axons. Moreover, the formation of myelin sheaths plays an important role in the nervous system functions. Thus, oligodendrocytes provide structural and electrical properties for axons by controlling their diameter as well as spacing and clustering of Na^+ channels at nodes and paranodes (Barres, 2008). Myelin represents an essential component of white matter in CNS, which contains approximately 40-50% myelin on a dry weight basis. Myelin dry weight consists of 70 % lipids and 30 % proteins (Baumann and Pham-Dinh, 2001). There are several specific proteins that we can find in myelin, including myelin basic protein (MBP), proteolipid protein (PLP) and myelin associated glycoprotein (MAG) (Ndubaku and de Bellard, 2008). Because of the high content of lipids and low content of water, myelin sheaths form an isolating layer and therefore enable fast nerve conduction (Baumann and Pham-Dinh, 2001). Myelinated axons appear as myelinated membrane segments, called internodes, and are separated by regions of unsheathed nerve membrane, termed nodes of Ranvier. There is an extremely high density of Na^+ channels in the node of Ranvier, which allows impulse to jump from

node to node. This fast transmission of impulse is driven by high resistance and low capacitance of myelin sheath (Shepherd, 1988, cit. by Baumann and Pham-Dinh, 2001). Oligodendrocytes are important in the development, maintenance and survival of axons and in control of growth of axon calibre (Baumann and Pham-Dinh, 2001). This is why injuries and demyelination, due to damage to oligodendrocyte, could lead to variety of serious diseases (Allen and Barres, 2009).

2.3.2 Oligodendrocyte progenitor cells (OPCs)

OPCs are multipolar cells that represent a big part of mammalian developing CNS and contact numerous small diameter pre-myelinated axons (Verkhratsky and Butt, 2007). There are different populations of OPCs that originate from different parts of CNS. These are specialized domains of spinal cord and forebrain, from where OPCs migrate long distances to their final destination, where they develop into mature oligodendrocytes (Bradl and Lassmann, 2010). In adult brain, OPCs persist in significant numbers and are in a relatively quiescence state, thus providing a potential source for new oligodendrocyres after injury (Wolswijk et al., 1990). Shi et al. (1998) found that adult OPCs in culture are not senescent cells and that they retain the capacity to divide rapidly. Studies have shown that rapidly proliferating OPC-like cells and endogenous precursor cells in the white matter of the brain can re-myelinate axons (Carroll and Jennings, 1994, cit. by Shi et al., 1998; Gensert and Goldman, 1997). These findings are important in demyelination injuries and diseases (Shi et al., 1998).

2.3.3 NG2-glia

NG2-glia are named after the proteoglycan Neuron glia 2, which they express (Stallcup et al., 2002). NG2 cells have been found in both grey and white matter and are considered as adult OPCs, as they provide re-myelinating oligodendrocytes following demyelination (Dawson et al., 2000). However, apart from their assumed functions as OPCs, NG2 cells have been identified as postmitotic complex cells with stellate morphology and are considered to be a distinct class of cells called synantocytes or polydendrocytes (Nishiyama et al., 2009). They have several physiological properties expressing voltage-gated ion channels and are involved in neural-glial interactions. Nevertheless, NG2 glia

interacts with nodes of Ranvier and formation of glial-scar, driven by CNS injuries (Butt et al., 2005).

2.3.4 Oligodendrocyte markers

Table 2: Oligodendrocyte markers

Marker	Function
O4	The monoclonal antibody O4 detects sulfatides and an unidentified sulfated glycoconjugate termed pro-oligodendroblast antigen (Bansal et al., 1999). Sulfatid is one of the major galactosphingolipid compounds of oligodendrocyte plasma membrane and myelin. It is also produced by Schwann cells in the peripheral nerve (Takahashi and Suzuki, 2012). In the early OPCs these sulfatid antigens appear to lack (Deng and Poretz, 2003). Sulfatide has the function of a negative regulator of oligodendrocyte differentiation (Hirahara et al., 2004), plays role in oligodendrocyte survival (Shroff et al., 2009) and also appears to function in myelin maintenance and axon structure (Marcus et al., 2006).
Sox10	Sox10 is one of the transcription factors of a large Sox family. It is characterized by DNA-binding and bending domain, called the high mobility group box (HMG box) (Kuhlbrodt et al., 1998). Sox10 plays a crucial role in neuronal crest development (Kim et al., 2003), peripheral gliogenesis (Ito et al., 2006), terminal oligodendrocyte differentiation and activation of several key myelin genes (Stolt et al., 2002). It has been reported that Sox10 also functions as an active nucleocytoplasmic shuttle protein, as it is entering and exiting the nucleus (Rehberg et al., 2002).
APC	Adenomatous polyposis coli (APC) is a 312 kD protein, and acts as a tumor suppressor. Its mutation reflects as familial adenomatous polyposis, which often develops into colon cancer (Lang et al., 2013). APC is expressed in oligodendroglial linage during normal oligodendrocyte development and it is important for oligodendrocyte regeneration and proliferation of OPCs. APC regulates oligodendrocyte differentiation trough β-catenin intracellular level regulation (Lang et al., 2013). In CNS it also regulates process formation, proliferation of radial glia, astrocytes, neurons and neuroblasts (Yokota et al., 2009; Imura et al., 2010).
Olig1	Oligodendrocyte transcription factor 1 (Olig1) is a basic helix-loop-helix transcription factor expressed exclusively in CNS (Zhou et al., 2000). Olig1 has roles in development and maturation of oligodendrocytes, evident especially within the brain (Lu, et al., 2002). It also has an important role in the repairing of demyelinating injuries (Arnett et al., 2004, cit. by Meijer et al., 2012).
Olig2	Oligodendrocyte transcription factor 2 (Olig2) is expressed very early in CNS development within the radial glia of the neural tube, which develop into motor neurons and mature oligodendrocytes (Malatesta et al., 2003; Tsai et al., 2012). Olig2, together with Olig1 also plays a dominant role in the pattering of the spinal cord (Meijer et al., 2012).
PDGFαR	Platelet-derived growth factor α receptor is a tyrosine kinase receptor and binds both A and B chains of platelet-derived growth factor (PDGF) (Mudhar et al., 1993). PDGFαR is in gliogenesis uniquely expressed by OPCs at very early stages of oligodendrocyte development and disappears at O4+ stage of oligodendrocyte maturation (Baumann and Pham-Dinh, 2001). PDGFαR and its ligands are important players in OPC proliferation and survival (Mudhar et al., 1993) and also necessary for the neuronal crest development and for normal pattering of the somites (Soriano, 1997).
NG2	NG2 is an integral membrane chondroitin sulfate proteoglycan with 260 kDa core protein. In adult CNS, NG2 is expressed together with PDGFαR, which proves the development in oloigodendrocyre linage (Baumann and Pham-Dinh, 2001). However, NG2 is not expressed in adult oligodendrocytes, astrocytes, microglia or neurons, proposing that the cells expressing NG2 antigen are a novel glial cell population (Dawson et al., 2000). Outside the nervous system, NG2 is expressed in many other tissues by different mesenchymal cell type. NG2 proteoglycan seems to contribute to proliferation and migration of immature progenitor cells and their responses to injuries such as inflammation or demyelination (Stallcup, 2002).

2.3.5 Oligodendrocyte pathology

The primary event in oligodentrocyte pathology is the breakdown of BBB which plays a big role in demyelinating diseases, such as MS (Baumann and Pham-Dinh, 2001). Secondly, oligodendrocytes have high metabolic activity and consequently use up large amounts of oxygen and ATP, and have a high iron content (McTigue and Tripathi, 2008). All this leads to the formation of reactive oxygen species, free radical formation and lipid peroxidation. Thus, oxidative stress, which can easily effects oligodendrocytes, is one of the mechanisms responsible for several neurological diseases, especially in the process of demyelination (Bradl and Lassman, 2010).

2.3.5.1 Multiple sclerosis

MS is one of the most common neurological diseases. It is a chronic, inflammatory disease of CNS and involves demyelination as a result of autoimmune attack on oligodendrocytes and myelin (Barres, 2008). Most patients with MS experience repeated attacks on oligodendrocytes and myelin via the inflammatory cascade that is activated repeatedly over time. In the early stages of demyelination in MS, re-myelination occurs due to generation of new oligodendrocytes and myelin formation. However, this repair mechanism fails in later, progressive stage of the disease and can no longer repair injured oligodendrocytes and depleted myelin, causing irreversible neurone damage to develop (Dhib-Jabult, 2007). It is not known whether the repair fails because of the exhaustion of the new OPCs, a deficiency in the relevant axonal signals or electrical activity that induces OPC proliferation and myelination, a diversion of OPCs into an astrocyte differentiation pathway, or the development of inhibitors that prevent migration or myelination by OPCs. The answer may lead to new drugs that promote myelin repair (Barres, 2008).

2.4 MICROGLIA

Microglia represent the immune system of the CNS and act as macrophages with the ability of phagocytosis. These cells originate from myeloid lineage, then enter the CNS and migrate all over the parenchyma where they transform into resting microglia. In case of insults of the nervous system, such as neurodegenerative diseases, strokes, traumatic injuries etc., microglial cells are activated (Heneka et al., 2010). With their processes they

rapidly converge on the site of injury without cell body movement, start to scan the microenvironment to remove damaged or dead cells and build a potential barrier between healthy and injured tissue (Davalos et al., 2005).

Microglial cells can secrete numerous cytokines and other pro-inflammatory mediators, including tumor necrosis factor α (TNFα), which plays a crucial role in promoting generation of new oligodendrocytes (Arrnet et al., 2001, cit. by Barres, 2008). There is a dendritic cell type of microglia, which has the capability of presenting myelin antigen to T cells within the brain (Miller et al., 2007). It has been shown that microglia also have an important role during the CNS development in eliminating inappropriate synaptic connections through classical complement cascade called complement component 1 q (C1q) (Stevens et al., 2007).

However, microglia are also involved in the progression of neurodegenerative diseases like PD and AD with their neuro-inflammatory mechanisms (Heneka et al., 2010). Cytokines, reactive oxygen species, complement factors, neurotoxic secretory products, free radical species and nitric oxide can contribute to neuronal dysfunction and cell death. Thus, activated microglia can be a potential target for future anti-inflammatory therapeutics (Sastre et al., 2006).

2.5 ADULT NEURAL STEM CELLS

Neural stem cells (NSCs) are present in the developmental brain and in the specific regions of postnatal and adult brain. These cells develop not only into neurons but also into glial cells (Alvarez-Buylla and Lim, 2004). Glial cells were primarily considered to have very different origin from nerve cells. Recent studies have shown that glial cells in development, radial glia, and subpopulations of astrocyte in adult mammals actually function as primary progenitors or NSCs (Kriegstein and Alvarez-Buylla, 2009).

In adult mammals, neurogenesis is most prominent in subventricular zone (SVZ) in the walls of lateral ventricles. The SVZ contains NSCs, also known as B cells, which are categorised as subpopulation of astrocytes as they are expressing GFAP, GLAST and other astroglial markers (Doetsch et al., 1997; Platel et al., 2009). B cells or SVZ astrocytes

maintain the epithelial organization with apical contact at the ventricle and basal endings in blood vessels. These cells give rise to actively proliferating C cells that function as intermediate progenitor cells (IPCs) or they keep amplifying progenitors in adult SVZ (Fig. 5) (Doetsch et al., 1999).

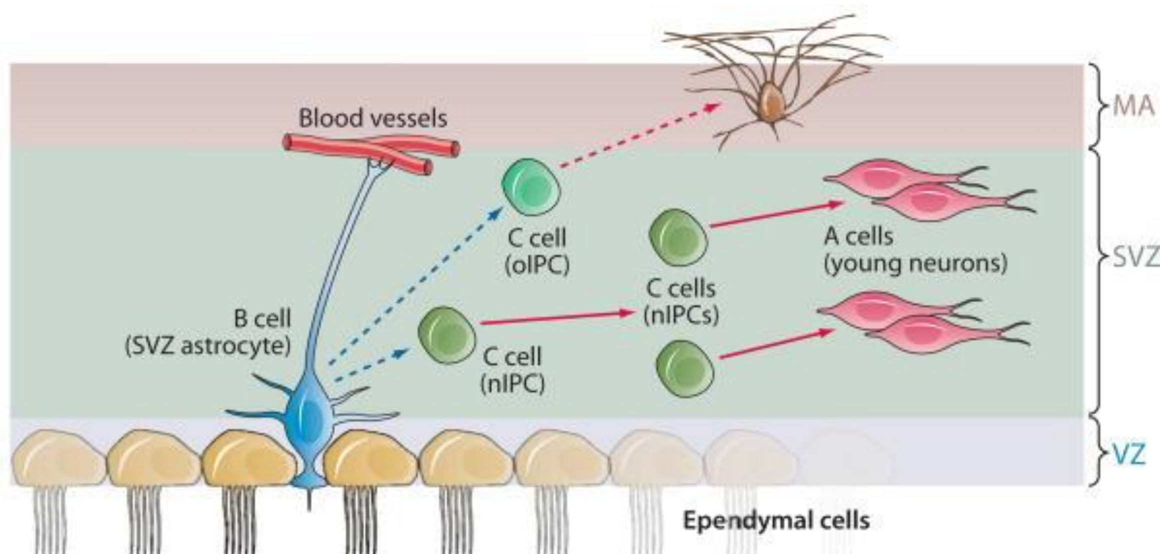

Figure 5: Schematic of progenitor types and lineages in the adult brain SVZ. NSCs of the wall of lateral ventricles of adult rodents correspond to type B cells. These cells generate neurons (A cells) through neurogenic intermediate progenitor cells (nIPCs) and give rise to oligodendrocyte through oligodendrocytic intermediate progenitor cells (oIPCs). Dashed arrows illustrate hypothetical modes of division: blue for asymmetric and red for symmetric divisions. VZ- ventricular zone, SVZ- Subventricular zone, MA- mantle (Kriegstein and Alvarez-Buylla, 2009)

SVZ astrocytes appear to act as adult NSCs and maintain characteristics of an adult stem cell since they regulate their proliferation and differentiate into specific types of neurons and glia, depending on their location in the brain (Kriegstein and Alvarez-Buylla, 2009). These NSCs may represent an important reservoir of precursor cells and produce a wide array of neuronal subtypes for brain repair, which leads to novel approaches for specific therapeutic applications (Lindvall and Kokaia, 2006).

2.6 Wnt/GSK-3β PATHWAY

Wnt signal molecules come from a large family of secreted glycolipoproteins. Wnt signalling regulates embryonic patterning, cell proliferation and determination, anatomy of the neuronal cytoskeleton, differentiation of synapses in cerebellum, as well as apoptosis (Patapoutian and Richardt, 2000). The canonical Wnt pathway is controlled by β-catenin.

Wnt binds to Frizzled and LDL receptor-related proteins 5 and 6 (LRP5/6) (He et al., 2004) and activates the cytoplasmic scaffolding protein Dishevelled (Dvl). The latter triggers the inhibition of glycogen synthase kinase-3β (GSK-3β), which results in stabilisation and nuclear translocation of β-catenin (Patapoutian and Richardt, 2000). Free β-catenin forms a nuclear complex with T-cell factor and lymphoid enhancer factor (TCF/LEF), DNA-binding transcription factors, activating the transcription of numerous gens (Fig. 6B) (Arce et al., 2006). In the absence of Wnt signalling, β-catenin is complexed with GSK-3β, Axin, APC and casein kinase 1 (CK1). GSK-3β and CK1 sequentially phosphorylate β-catenin, which drives β-catenin ubiquitination and consequently its degradation by proteasomes (He et al., 2004). Wnt target genes are repressed by inhibitors Groucho/transducin-like enhancer (Gro/TLE) and histone deacetylases (HDAC) (Fig. 6A) (Daniels and Weis, 2005).

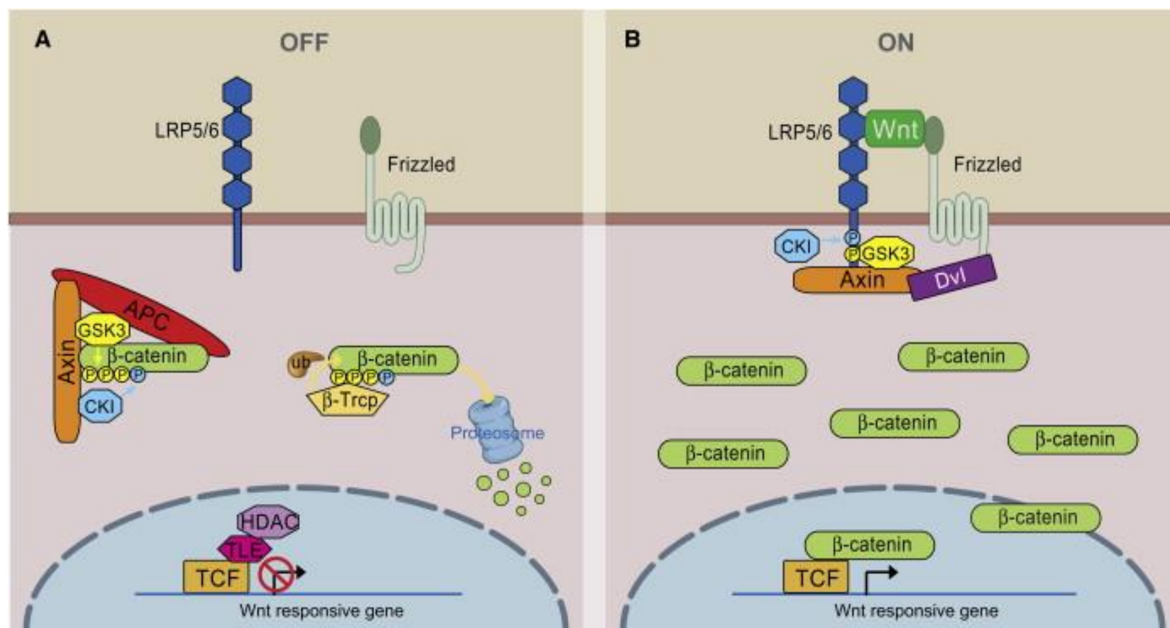

Figure 6: Wnt/β-catenin signalling. A) The absence of Wnt signal molecule. B) The presence of Wnt signal molecule. LRP5/6- low-density lipoprotein receptor related proteins 5 and 6, APC- adenomatous polyposis coli, GSK3- glycogen synthase kinase-3β, CK1- casein kinase 1, ub- ubiquitin, β-Trcp- E3 ubiquitin ligase, HDAC- histone deacetylase, TLE- transducin-like enhancer, TCF- T-cell factor, Dvl- Dischevelled (MacDonald et al., 2009)

Hence, GSK-β3 by its actions through regulating Wnt signalling, is an essential molecule for proliferation and differentiation signals and cell fate determination. Inhibition of GSK-3β with several inhibitors (ARA-014418, lithium, indirubin and L803-mt) results in

increased OPCs proliferation and survival, increased oligodendrocyte differentiation and myelination. Furthermore, GSK-β3 inhibition has positive effects on oligodendrocyte regeneration and re-myelination, which may give options to develop new therapeutics for demyelinating disease, e.g. MS (Azim and Butt, 2011). Inhibition of GSK-β3 signalling with lithium also has an effect on astrogliosis. It results in generation of a novel astrocyte, different from reactive, scar astrocytes. This may be an important factor in the repairment of CNS injuries and diseases (Rivera, 2014).

2.7 OPTIC NERVE

The optic nerve is the second pair of cranial nerves that arise from the retina and carry visual information to the brain. It is the only cranial nerve that is a part of CNS, whereas the remaining eleven are peripheral nerves. Thus, the optic nerve may be used as a model of CNS tract, which includes axons of retinal ganglion cells together with oligodendrocytes, type 1 and type 2 astrocyte (Raff, 1989), NG2-glia and microglia (Butt et al., 2005). The rodent optic nerve is easy to isolate and represents a suitable model for studying axonal-glial interactions in their natural state, since there are no neuronal cell bodies. Furthermore, it is also an ideal preparation for the study of myelination of CNS axons, relationships between myelination and axonal conduction properties, and physiological characteristic of glial cells (Bolton and Butt, 2004).

In the early 1980s, oligodendrocyte-type 2 astrocyte bipotential progenitors (O-2A) were first discovered incultures of the optic nerve by Raff and colleagues. O-2A cells in culture develop into type 2 astrocytes if cultured in media with fetal calf serum (FCS), and into oligodendrocytes if cultured in serum free media (Raff et al., 1984). In the absence of evidence for type-2 astrocytes *in vivo*, O-2A cells have been referred to as OPCs (Butt et al., 2005). All this leads to an important aspect of the approach of *in vitro* culturing, as we can manipulate the cells and their environment. By exposing them to a variety of signals, the full differentiation potentiality and characterisation can be explored (Hardy and Reynolds, 1991).

2.8 *In vitro* CULTURING OF GLIAL CELLS FROM ADULT MOUSE CNS

Much of our understanding of glial cell biology comes from the studies of glial cell lines or cells in cultures that have been isolated from embryonic or early postnatal brain (McCarthy and de Vellis, 1980; Dincman et al., 2012; Chen et al, 2007; Emery and Dugas, 2013). McCarthy and de Vellis (1980) contributed an important advance in the development of astrocyte and oligodendrocyte cultures preparation from rodent neonatal brain. However, the properties of glial cells isolated from prenatal brain show an unclear relationships to those of mature glia in the adult brain (Cahoy et al. 2008). Furthermore, it has become clear that it is very difficult to study mature astrocytes, OPCs and oligodendrocytes in culture, particularly in pathology/neuro-degeneration and regeneration. There have been only a few previous studies that isolated pure glial cell populations from adult rodent CNS (Foo et al., 2011; Cahoy et al., 2008; Shi et al., 1998).

An important aspect of studying glial cells, their functions, involvement in neurodegenerative diseases and their potential treatments in mouse, is the use of the 3Rs principle:

- REPLACEMENT: the use of non-animal methods such as cell cultures, human volunteers and computer modelling instead of animal to achieve scientific aim
- REDUCTION: the use of methods that enable researchers to obtain comparable amounts of information from fewer animals or more information from the same number of animals
- REFINEMENT: the use of methods that alleviate or minimise potential pain, suffering or distress, and that enhance animal welfare for those animals that cannot be replaced (Robinson, 2005)

To achieve these principles it is important to develop stable glial populations from adult mouse CNS *in vitro*. That is how we can replace and reduce the use of *in vivo* methods. In addition, as neurons depend on astrocytes and oligodendrocytes for their survival, it has not been possible to get their functional roles *in vivo* simply by deleting them, which makes culture studies a powerful approach (Foo et al., 2011). However, there are still some limitations, which come along with different isolation and culturing techniques, and can

result in genomic instability and viability. Thus, it is significant to provide *in vitro* findings that will be better recapitulated *in vivo* (Dincman et al. 2012).

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Animals

Mice aged between postnatal day (P)3 and P7, and adults were used throughout. The wild type mouse strain used was C57BL/6 strain. Transgenic mouse line was used, GFAP-eGFP, in which enhanced green fluorescent protein (eGFP) is under control of the glial-specific promotor GFAP. All research involving animals was approved by the University of Portsmouth Ethics Committee and by the Home Office of the United Kingdom under the Animals Scientific Procedures Act, 1986. Animals were killed humanely by cervical dislocation, and brains and optic nerves were removed rapidly and placed in saline.

3.1.2 Agents

Table 3: List of chemicals

Chemical	Abbreviation	Supplier
Phosphate Buffered Saline tablets	PBS	Sigma
Triton X-100	Triton	Sigma
Poly-L-lysine	Poly-L-lysine	Sigma
Laminin	Laminin	Sigma
Ethanol	Eth	Fisher Scientific
Distilled water	dH_2O	Gibco
Hanks' Balanced Salt Solution (1x), $+CaCl_2$ $+MgCl_2$	HBSS $+CaCl_2$ $+MgCl_2$	Gibco
Hanks' Balanced Salt Solution (1x), $-CaCl_2$ $-MgCl_2$	HBSS $-CaCl_2$ $-MgCl_2$	Gibco
Trypsin	Trypsin	Sigma
Deoxyribonuclease I	DNAse I	Sigma
Fetal Bovine Serum	FBS	Gibco
Neuron Goat Serum	NGS	Gibco
Horse serum	HS	Gibco
Penicillin/Streptomycin	Pen/strep	Sigma
Glucose	Glucose	Sigma
Opti-Minimal Essential Medium	Opti-MEM	Gibco
Advanced Dulbecco's Modified Eagle Medium/F12	Advanced DMEM/F12	Gibco
Neurobasal- A Medium (1x)	NB-A Medium	Gibco
L-glutamin	L-glutamin	Sigma
B27 suplement	B27	Millipore
Fibroblast growth factor 2	FGF2	Millipore
Recombinant Human Platelet-derived Growth Factor AA	rhPDGF-AA	R&D Systems
Bovine Serum Albumin	BSA	Sigma
N2 suplement	N2	Millipore
Sodium hydrogen carbonate	$NaHCO_3$	Sigma-Aldrich

continued

continuation of Table 3. List of chemicals

Chemical	Abbreviation	Supplier
Paraformaldehyde	PFA	TAAB
Hoechst	Hoechst	Invitrogen
Fluoromount	Fluoromount	Sigma
Neural Tissue Dissociation Kit (P)	NTDK (P)	Miltenyi Biotec
Neural Tissue Dissociation Kit (T)	NTDK (T)	Miltenyi Biotec
Anti-O4 Micro Beads: human, mouse, rat	Anti-O4 Micro Beads	Miltenyi Biotec

Table 4: Primary antibodies

Antigen	Host	Dilution	Supplier
GFAP	chicken	1:300	Millipore
GLAST	rabbit	1:1000	Abcam
Sox10	rabbit	1:300	Millipore
PDGFαR	rabbit	1:300	Santa Cruz Biotechnology
O4	mouse	1:50	R. Reynolds, ICL, UK

Table 5: Secondary antibodies

Conjugate	Reactivity	Dilution	Supplier
Alexa Fluor 488	goat anti-rabbit	1:500	Invitrogen
Alexa Fluor 568	goat anti-rabbit	1:500	Invitrogen
Alexa Fluor 568	goat anti-chicken	1:500	Invitrogen
Alexa Fluor 488	goat anti-mouse	1:500	Invitrogen
Alexa Fluor 568	goat anti-mouse	1:500	Invitrogen
TRITC	goat anti-mouse	1:100	Sigma

Table 6: Culture media recipes

NSC media	50 %	Opti-MEM
	25 %	HS
	25 %	HBSS +$CaCl_2$ +$MgCl_2$
	1 %	Pen/strep
	1 %	Glucose 50 %
NBA-A media	97 %	NB-A
	2 %	B27
	1 %	L-glutamin
	0.5 %	Pen/strep
OPC-A media	95 %	DMEM/F2
	2 %	B27
	1 %	Pen/strep
	1 %	N2
	0.01 %	BSA
	2.1 g/L	$NaHCO_3$
	40 ng/mL	FGF2
	20 ng/mL	rhPDGF-AA

Media was filter sterilized through 0.22 micron filter system to prevent contamination. Media was incubated for 15 min in 37 ˚C, 5 % CO_2 incubator before use and was stored at 4 °C if not used.

3.1.3 Equipment

Table 7: List of equipment

Equipment	**Supplier**
Automatic pipetts	Gilson
Pipettor	Fisher Brand
Laminar flow cabinet (class II)	NuAire
CO_2 incubator	Jouan
Centrifuge, model 1k15	Sigma
Centrifuge, model 5416	Eppendorf
Confocal laser scanning microscope, model 510	Zeiss
Cell counter, Vi-Cell XR	Beckman Coulter
24-well plates	Cellstar
MACS Pre-Separation Filters, 70 µm	Miltenyi Biotec
MACS Separator	Miltenyi Biotec
MACS MS Column	Miltenyi Biotec

3.2 METHODS

3.2.1 Dissection of mouse brain and optic nerve

Pieces of mouse forebrain, cerebellum or 6 optic nerves were collected from adult mice. Whole brains were harvested from pups. The brain was collected by cutting the skin and skull along the midline and reflected to expose the brain. For the optic nerves, the eyeballs were cut off and the brain was moved to expose and cut the optic nerves on their opposite side. Brain samples were placed into 1 ml and optic nerves into 500 µl of HBSS -$CaCl_2$ -$MgCl_2$.

3.2.2 Tissue dissociation of mouse brain and optic nerve

3.2.2.1 In-house dissociation method

In-house dissociation method is based on enzymatic dissociation with trypsin, which breaks down the integrity of the tissue by degradation of extracellular adhesion proteins. This method was used for the dissociation of brain and optic nerve of adult mouse.

The protocol for in-house dissociation method is described below:

1. An appropriate volume of dissociation solution was first prepared. The dissociation solution for the optic nerve was prepared in 2 mL microcentifuge tube, while 15 mL tube was used for the preparation of solution for brain dissociation. The chemical compounds of dissociation solution and their volumes are listed below (Tab. 8). The solution was prepared under the laminar flow hood to prevent contamination and was preheated in 37 °C incubator until ready to use.

Table 8: Dissociation solution for brain and optic nerve

Dissocation solution compounds	**Brain**	**Optic nerve**
HBSS -$CaCl_2$ -$MgCl_2$	1.96 mL	980 µL
Trypsin	40 µL	20 µL
DNAse I	12 µL	6 µL
FBS	114µL	57 µL

2. For tissue collection, appropriate volume of HBSS -$CaCl_2$ -$MgCl_2$ was pre-prepared and preheated for 10 min in 37 °C incubator.
3. The collected optic nerves were first cut into small pieces for more effective cell dissociation. For brain dissociation, the brain tissue was placed in a fresh petri dish. To prevent the tissue to dry out and for the later easier re-collection of the tissue, 1 mL of HBSS -$CaCl_2$ -$MgCl_2$ was added. The tissue sample was then cut with a scalpel in approximately 1 mm^3 pieces and pipetted back into the tube. The petri dish was rinsed with additional 1-2 mL of HBSS -$CaCl_2$ -$MgCl_2$ to collect any remaining tissue.
4. The preheated dissociation solution was added to the tissue sample and incubated for 45 min in 37 °C, 5 % CO_2 incubator. The tube was inverted every 10 min to keep the pieces in the solution.
5. After the incubation, the optic nerve sample was dissociated mechanically by pipetting slowly up and down 10 times with each of P200, P10 and 1 mL sterile pastette, respectively, avoiding making air bubbles. For easier brain dissociation, P1000 was used first in order to break down bigger pieces of the brain tissue.
6. The mechanically dissociated tissue sample was centrifuged at 300 x g for 2 min at room temperature (RT).
7. The supernatant was carefully removed. 500 µL of HBSS +$CaCl_2$ +$MgCl_2$ was added to the cells dissociated from the optic nerve and 1 mL to the cells dissociated from the brain.
8. 70 µm strainer was pre-wetted with 2 mL of HBSS +$CaCl_2$ +$MgCl_2$.
9. Cell suspension was filtered through the strainer to remove cell aggregates or other large particles. The tube with the tissue was then rinsed with 1-2 mL of HBSS +$CaCl_2$ +MgCl2 and added to the strainer.
10. The filtered suspension was then centrifuged at 300 x g for 10 min at RT.
11. The supernatant was carefully aspirated and 1 mL of pre-heated media was added.
12. A small aliquot of cells was removed to determine the cell number and viability with the cell counter. In the meantime the cells were kept on ice.

3.2.2.2 Neural Tissue Dissociation Kit (P) and (T)

The Neural Tissue Dissociation Kits (NTDKs) have been designed for the dissociation of the neural tissue to single-cell suspensions by enzymatic degradations of extracellular adhesion proteins. NTDK (P) contains papain, and NTDK (T) contains trypsin. Some antigen epitopes are damaged by papain, others by trypsin. The papain based kit was used as a tissue dissociation method to gain oligodendrocytes, since oligodendrocyte specific O4 antigen is thus preserved . The kit based on trypsin was used in order to obtain cells with astrocyte specific GLAST antigen. This dissociation kit method was used for both brain and optic nerve tissue dissociation of adult mouse and pups.

The protocol for NTDKs is described below:

1. An appropriate volume of Enzyme Mix I was first prepared. 1950 µL of Enzyme Mix I corresponds up to 400 mg of tissue. Solution I was added to Solution II and was briefly vortexed. This was then preheated in 37 °C, 5 % CO_2 incubator for 15 min before use. Volumes of reagents used are listed below (Tab. 9).

Table 9: Enzyme Mix I for NTDK (P) and (T) tissue dissociation

Enzyme Mix I	Solution I	Solution II
NTDK (P)	50 µL	1900 µL
NTDK (T)	200 µL	1750 µL

2. For tissue collection, appropriate volume of cold HBSS -$CaCl_2$ -$MgCl_2$ was pre-prepared. The weight of the brain was measured to assure the collected tissue was not heavier than 400 mg.
3. For more affective cell dissociation, the optic nerves were cut into small pieces. The brain sample was transfered into a fresh petri dish. To prevent the tissue to dry out and for later easier re-collection of the tissue, 1 mL of HBSS -$CaCl_2$ -$MgCl_2$ was added. The brain tissue was then cut with scalpel in approximately 1 mm^3 pieces and pipetted back into the tube. Petri dish was rinsed with 1-2 mL of HBSS -$CaCl_2$ -$MgCl_2$ to collect any remaining tissue.

4. The tissue sample was centrifuged at 300 x g for 2 min at RT.
5. The supernatant was carefully aspirated, which was followed by the addition of a pre-heated Enzyme Mix I to the pellet, and gently mixed to avoid making air bubbles.
6. The tissue was then incubated at 37 °C, 5 % CO_2 for 15 min in incubator.
7. During the incubation, Enzyme Mix II was prepared by adding Solution 3 to Solution 4. Volumes of reagents used were appropriate for up to 400 mg of tissue and are listed below (Tab. 10).

Table 10: Enzyme Mix II for NTDK (P) and (T) tissue dissociation

Enzyme Mix II	Solution 3	Solution 4
NTDK (P)	20 µL	10 µL
NTDK (T)	20 µL	10µL

8. After the incubation, Enzyme Mix II was added to the tube with the tissue and gently inverted. The tissue was then mechanically dissociated by pipetting slowly up and down 10 times with P1000, avoiding making air bubbles.
9. The mechanically dissociated tissue was incubated in 37 °C, 5 % CO_2 incubator for 10 min.
10. After the second incubation, the optic nerve tissue was dissociated mechanically by pipetting slowly up and down 10 times with each of P200, P10 and 1 mL sterile pastette, respectively, avoiding making air bubbles.
    For easier brain dissociation, P1000 was used first in order to break down the remaining bigger pieces of the brain tissue.
11. 70 µm strainer was pre-wetted with 2 mL of HBSS $+CaCl_2$ $+MgCl_2$.
12. Cell suspension was filtered through the strainer to remove cell aggregates or other large particles. The tube with the tissue was then rinsed with 1-2 mL of HBSS $+CaCl_2$ +MgCl2 and added to the strainer.
13. The cell suspension was then centrifuged at 300 x g for 10 min at RT.
14. After the centrifugation, the supernatant was carefully aspirated and pellet was re-suspended in 4 mL of HBSS $+CaCl_2$ +MgCl2, which was followed by a second centrifugation at 300 x g for 10 min at RT.

15. The supernatant was carefully aspirated. The cells were re-suspended in 1 mL of pre-heated media.

16. A small aliquot of cells was removed to determine the cell number and viability with cell counter. In the meantime the cells were kept on ice.

3.2.3 Magnetic Activated Cell Sorting (MACS) of O4$^+$ cells

The principle of MACS Separation is magnetic labelling, e.g. O4$^+$ cells with Anti-O4 MicroBeads. Then the cell suspension is passed through a MACS Column, which is placed in the magnetic field of the MACS Separator. The magnetically labelled O4$^+$ cells are retained in the column and the unlabelled cells run through. After removing the column from the magnetic field, O4$^+$ retained cells can be eluted from the column as a positively selected cell fraction (Fig. 7).

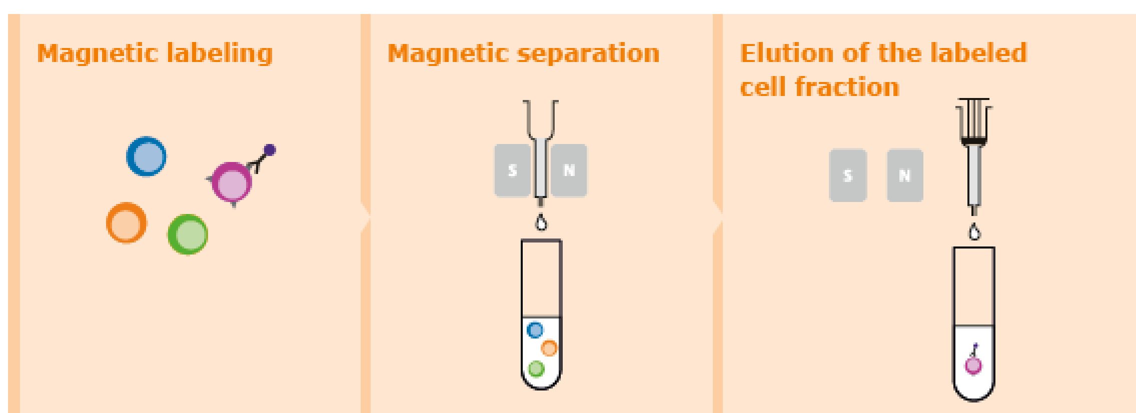

Figure 7: Principle of the MACS Separation (MACS Miltenyi Biotec, 2014)

3.2.3.1 Magnetic labelling

1. After the determination of the total number of dissociated cells, not more than 10^7 cells were taken from the cell suspension and centrifuged at 300 x g for 10 min.
2. The supernatant was carefully aspirated. 90 µL of cold BSA 0.5 % and 10 µL of Anti-O4 MicroBeads were added to the pellet. Cells were re-suspended thoroughly by pipetting slowly up and down.
3. The labelled cells were incubated for 15 min at 4°C in the dark.

4. After the incubation, the cells were washed by adding 1 mL of BSA 0.5 % and pipetted slowly up and down. They were then centrifuged at 300 x g for 10 min.
5. The supernatant was carefully aspirated and the cells were re-suspended in 500 µL of BSA 0.5 %.

3.2.3.2 Magnetic separation

1. During the centrifugation, the MACS column was placed on the MACS magnetic stand. The column was rinsed three times with 500 µL of BSA 0.5 %.
2. Cell suspension was applied onto the column. Flow-through with unlabelled cells was collected.
3. The column was rinsed three times with 500 µL of BSA 0.5 %
4. The column was then removed from the magnetic field and placed onto a fresh 2 mL tube. 500 µL of BSA 0.5 % was added onto the column. The magnetically labelled cells were immediately flushed out by the push of plunger into the column.
5. Isolated cells were then centrifuged at 300 x g for 10 min.
6. The supernatant was carefully aspirated. Cells were re-suspended in 1 mL of pre-heated media.
7. A small aliquot of isolated cells was removed to determine the cell number and viability with the cell counter. The number of cells in flow-through was also determined. In the meantime the cells were kept on ice.

3.2.4 Cell culturing

A day before the cell dissociation and isolation, cover slips were prepared:

1. 13 mm cover slips were first dipped into ethanol to avoid contamination. Each of the cover slips was then placed in a 24 well plate and left resting against the side of the well to dry out.
2. Poly-L-lysine was diluted in sterile double distilled water in ratio 1:100. 100 µL of diluted lysine was pipetted onto the centre of each cover slip and left overnight at 37 °C, 5 % CO_2 in an incubator.

3. The following day, laminin (1 mg/mL) was diluted in sterile double distilled water in ratio 1:200.
4. Lysine was removed and 25 µL of diluted laminin was pipetted onto the centre of each cover slip and left for 2h at 37 °C, 5 % CO_2 in an incubator.
5. After the incubation, the laminin was replaced with 30 µL of distilled water, if clover slips were not needed straight away.

After the dissociation and isolation of the cells, approximately 10^6 cells/cm^2 were plated onto the centre of each pre-prepared cover slip. Approximately 1 mL of preheated media was added carefully to each well with cells. The cells were kept in culture at 37 °C, 5 % CO_2 for 5-7 days. The media was changed every 3-4 days.

3.2.5 Immunohistochemistry

1. **Fixation**
   After 5-7 days in culture, the media was carefully aspirated from the wells with cells. The cells were fixed with 4 % PFA and incubated for 10 min at 37 °C in the incubator. PFA was removed and the cells were washed two times for 10 min in PBS.
2. **Blocking**
   The blocking solution was prepared with 10 % NGS in PBS for cell surface antigens and in PBS with 0.01 % triton (PBS-T) for non-surface antigens. Solution was added to each well with cells and incubated at 4 °C for 1h in the dark.
3. **Primary antibodies**
   The cells were incubated overnight in the dark at 4 °C with primary antibodies in 10 % NGS in PBS or PBS-T solution.
4. **Secondary antibodies**
   The following day, the cells were washed three times for 10 min in PBS to remove all unlabelled primary antibodies. The cells were then incubated for 1h in the dark at RT with secondary antibodies in 10 % NGS in PBS or PBS-T solution. Negative controls for antibody staining did not include primary antibodies to show the specificity of the secondary antibodies.

5. **Mounting**

   After the incubation with secondary antibodies, the cells were washed three times for 10 min in PBS. Approximately 10 µL of mounting media was added onto a glass slide. The cover slip with cells was then carefully taken from the well and placed onto the mounting media with the cells facing down. Slides were left to dry and stored at 4 °C.

6. **Confocal microscopy**

   All cell images were captured with confocal laser scanning microscope and edited in Zeiss LSM Image Browser software.

4 RESULTS

4.1 DISSOCIATED GLIAL CELS OF ADULT MOUSE CNS

Cell dissociation experiments were focused on the optic nerve of adult mouse, where in-house and NTDK dissociation methods were tested. Two different media were used for cell culture for comparison, one with serum (NSC media) and the other with serum free media (NB-A media). The dissociation of forebrain and cerebellum, using NTDK method, were initially performed as positive controls.

4.1.1 Dissociated cells of optic nerve

Using the in-house method, approximately 0.6 x 10^7 cells/sample from optic nerves of adult wild type (Wt) mouse were dissociated, with the viability of about 93 %. The in-house method proved to be a successful technique for glial cell dissociation of adult optic nerve tissue, as the cells were capable of being maintained in culture for 7 days (Fig. 8). Both types of glial cells, astrocytes and oligodendrocytes, were detected by their specific markers. An image of cells characteristic of fibrous astrocytes was captured, expressing the astrocyte specific GFAP marker (Fig. 8A-D). Cells with the origin of oligodendrocyte lineage were detected by the Sox10 marker (Fig. 8E-H); the Sox10 transcription factor is specific for oligodendrocyte lineage cells and is expressed by OPC and oligodendrocytes, and the cells identified here had the characteristic bipolar morphology of early OPC (or O-2A) in culture.

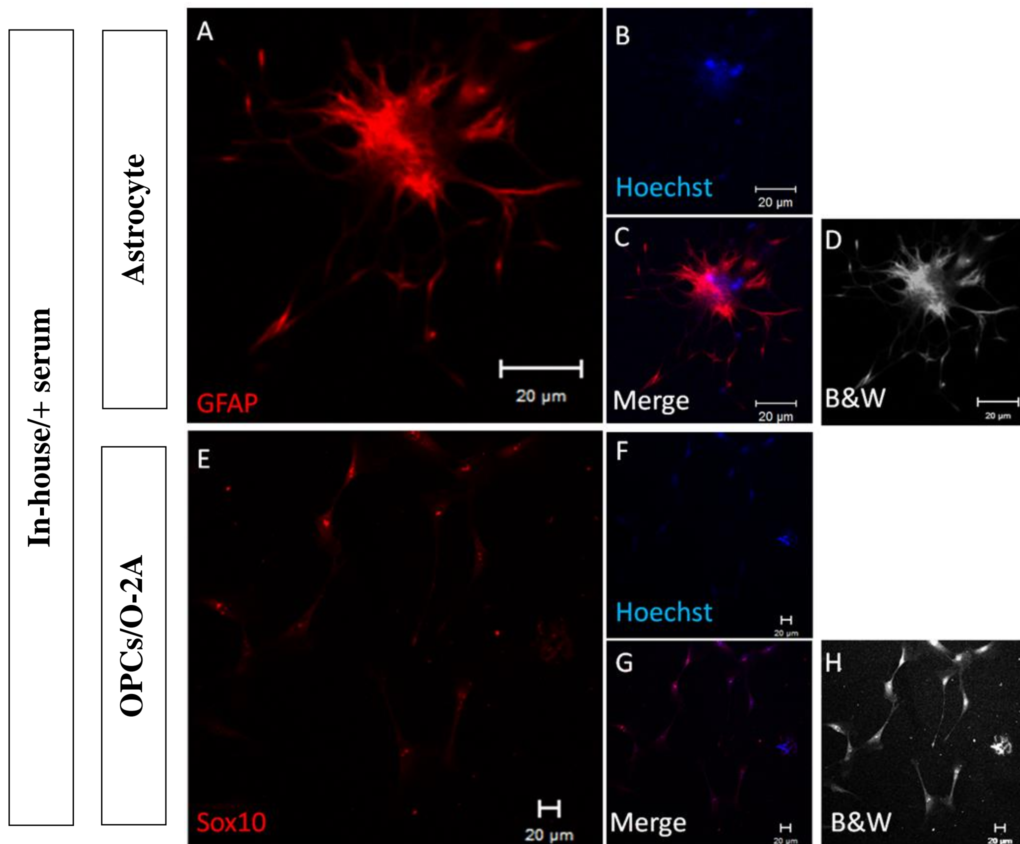

Figure 8: Dissociated glial cells from the optic nerve of adult mouse, using in-house dissociation method. (A-D) Cells expressing astrocyte specific GFAP marker and (E-H) cells expressing oligodendrocyte specific Sox10 marker. Cells were cultured 7 days *in vitro* in media with serum. B&W- black and white; scale bars = 20µm, 20x

Using the NTDK method, approximately 0.4 x 10^7 cells/sample was dissociated from adult Wt mouse optic nerve, with viability of about 93 %. The NDTK method, which is originally intended for the dissociation of mouse brain tissue (MACS Miltenyi Biotec, 2011), was found to be transferable to the adult mouse optic nerve tissue, considering that the cells were able to be maintained in culture for 7 days (Fig. 9). This method was additionally used because of its compatibility with the MACS isolation system. With the same purpose we focused on testing GLAST and O4, to see whether they are good markers for the continuation of cell isolation with Anti-O4 MicroBeads and Anti-GLAST (ACCSA-1) MicroBead Kit. Because of lack of time, we later decided to concentrate only on $O4^+$ cell isolation with Anti- O4 MicroBeads, and we therefore continued using papain based kit for oligodendrocyte dissociation.

Kit dissociated cells expressed both oligodendrocyte and astrocyte specific markers (Fig. 9). O4 proved to be an appropriate oligodendrocyte marker as it was co-expressed with another oligodendrocyte specific marker, Sox10 (Fig. 9A-F). The same applies for GLAST as an astrocyte marker, which was expressed along with GFAP (Fig. 9G-L).

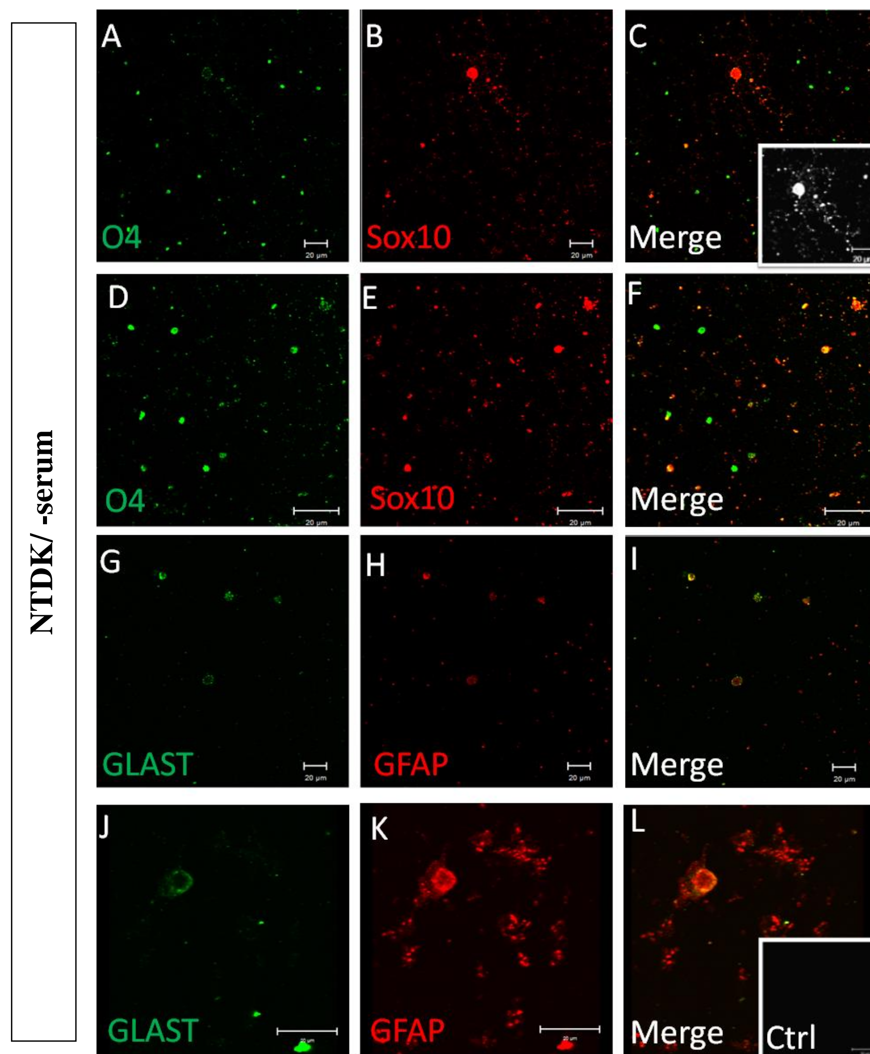

Figure 9: Dissociated glial cells from the optic nerve of adult mouse, using NTDK dissociation method. (A-F) Cells expressing oligodendrocyte O4 and Sox10 marker. (G-L) Expression of astrocyte GLAST and GFAP marker. All cells were cultured 7 days *in vitro* in serum free media. Ctrl- control; scale bars = 20 µm, 40x

Comparing both methods of optic nerve dissociation, they turn out to be equally successful by means of keeping cells in culture for 7 days (Fig. 10). By using the media with the serum, we wanted to stimulate the development of astrocyte, and by using the serum free media, that of oligodendrocytes (Raff et. al, 1984). With the serum media we managed to

culture GFAP positive cells, which refer to astroglia (Fig. 10A-D). On the other hand, we obtained oligodendroglia by culturing the dissociated cells in the serum free media and detected expression of oligodentrocyte O4 and Sox10 markers (Fig. 10E-H). However, it is still likely to get cells positive for oligodendrocytes when cultured in serum media (Fig. 8E-H) and vice versa; cells positive for astrocyte markers in serum free media (Fig. 9G-L).

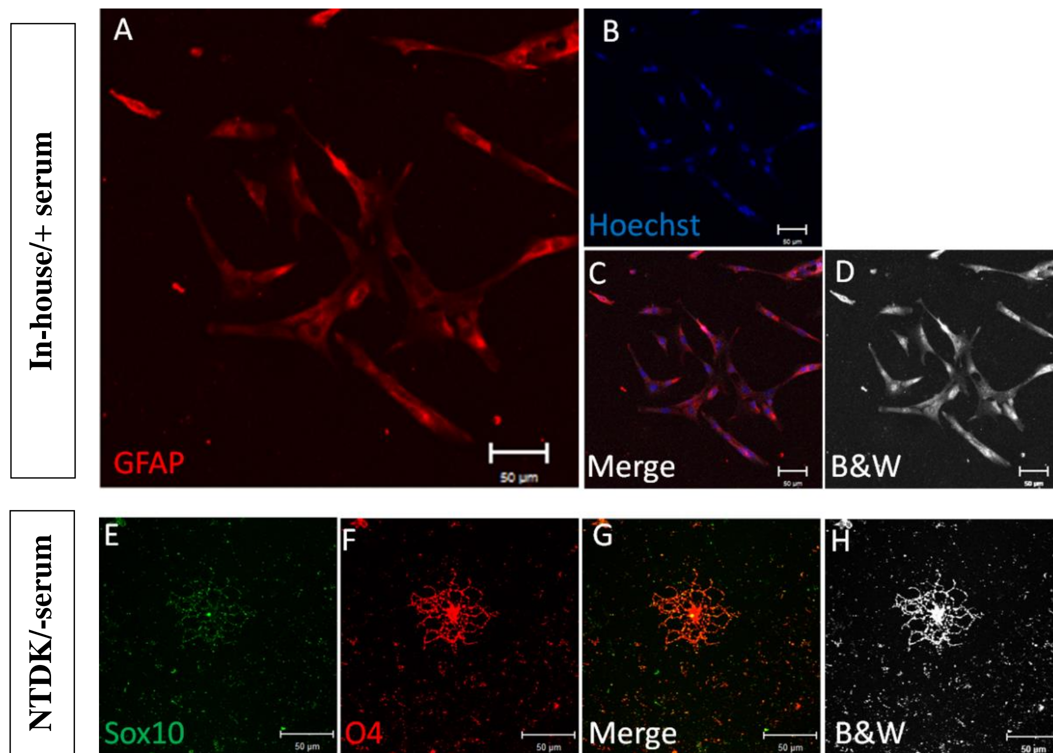

Figure 10: Comparison of in-house and NTDK dissociation method. (A-D) Astrocytes, expressing GFAP marker, dissociated with in-house method, 20x. (E-H) Image of oligodendrocyte, expressing Sox10 and O4 markers, dissociated with NTDK method, 40x. All cells were cultured 7 days *in vitro*. Scale bars = 50 µm

4.1.2 Dissociated cells of forebrain

The total cell number of cells dissociated from adult forebrain depends on the size of the tissue sample collected. Approximately 8 x 10^7 cells/sample was dissociated from adult forebrain, with viability of about 93 %, using NTDK dissociation method. The dissociation of the adult mouse forebrain was primarily used as a control to the optic nerve dissociation. NTDK method was found to be suitable for the adult forebrain tissue dissociation and provided us with a sufficient number of cells for further MACS cell isolation. Despite the serum free media we captured $GLAST^+$ and $GFAP^+$ astrocytes. These markers resulted as good markers for the detection of astroglia (Fig. 11).

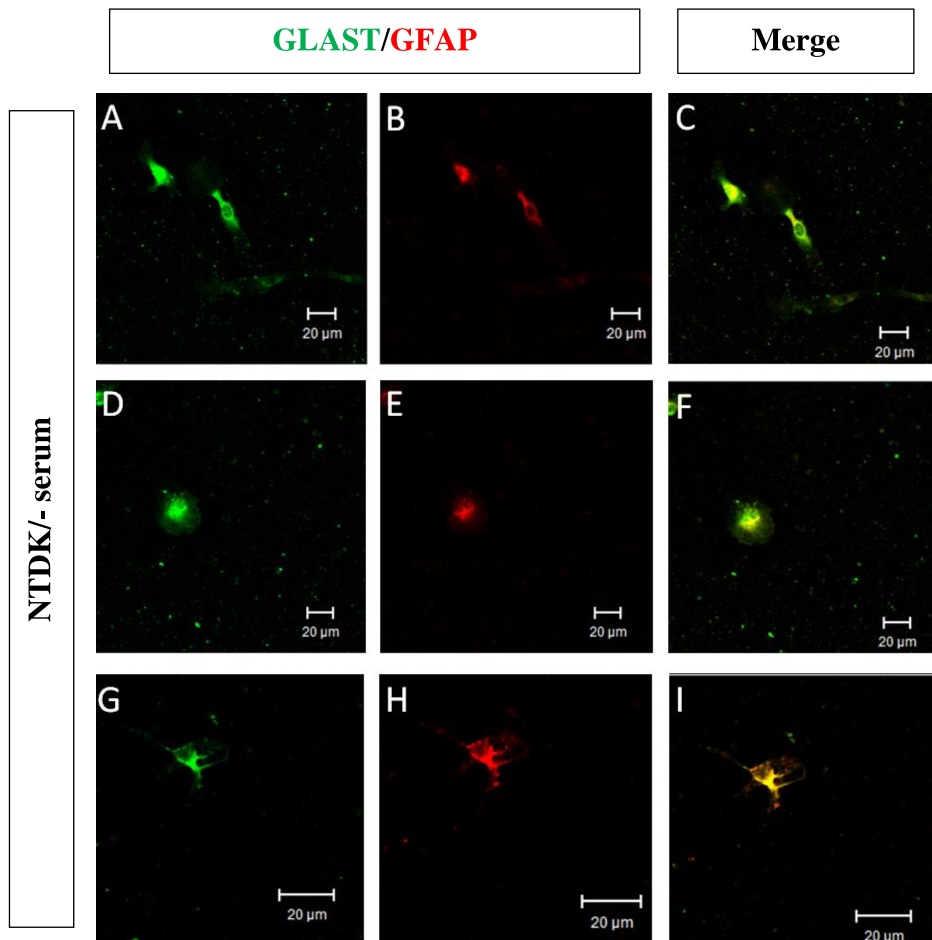

Figure 11: Dissociated astrocytes from adult mouse forebrain. (A-I) Astrocytes, expressing GLAST and GFAP markers. Cells were cultured for 7 days *in vitro* in serum free media. Scale bar = 20 µm

Interestingly, immunolabelling with PDGFαR primary antibodies was expressed along with a strong astroglial GFAP marker (Fig. 12). This is characteristic of O-2A cells, which have a bipolar morphology and co-expressed OPC and GFAP markers (inset, Fig. 12F). Not all cells co-express GFAP and PDGFαR (Fig. 12A-C), indicating mixed populations of astrocytes, OPC and O-2A cells; immunolabelling was not observed in the negative control (inset, Fig. 12C).

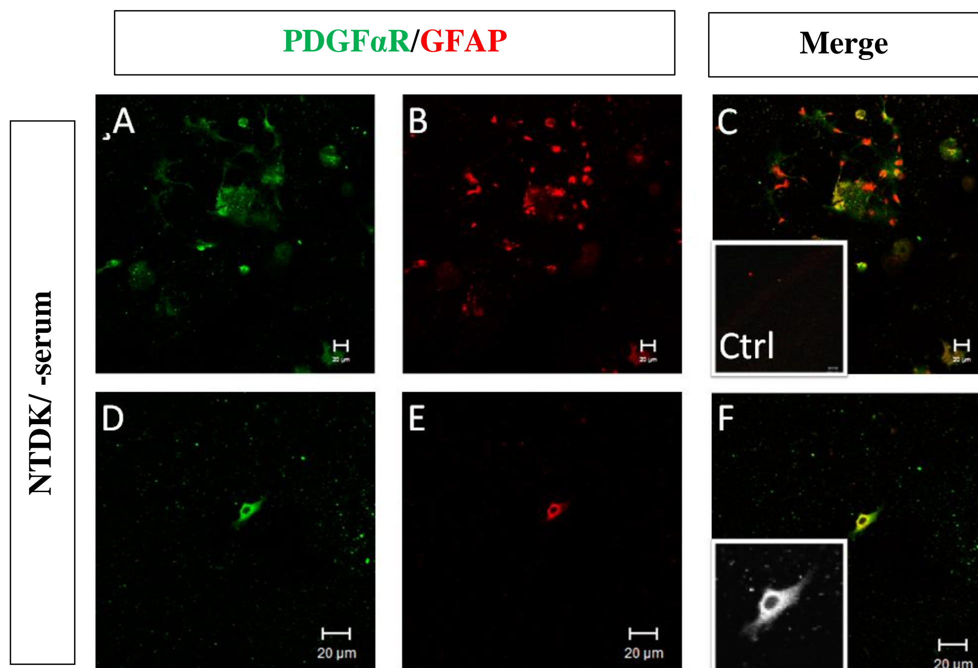

Figure 12: Dissociated glial cells from adult mouse forebrain. (A-F) Co-expression of PDGFαR and GFAP markers, representing O-2A cells. Cells were cultured for 7 days *in vitro* in serum free media. Ctrl- control; scale bar = 20 µm, 20x

4.1.3 Dissociated cells of cerebellum

Approximately 5 x 10^7 cells/sample was dissociated from adult GFAP-eGFP transgenic mouse cerebellum, with viability of about 89 %. Transgenic mice were used in order to prove the ability of survival of transfected cells after they undergo the cell dissociation process. Results showed that the dissociation has no effect on transfected cells, which were able to continue expressing transfected GFP gene (Fig. 13A, E, H). Once again, there were examples of co-expression of PDGFαR and GFAP (Fig. 13 E-G).

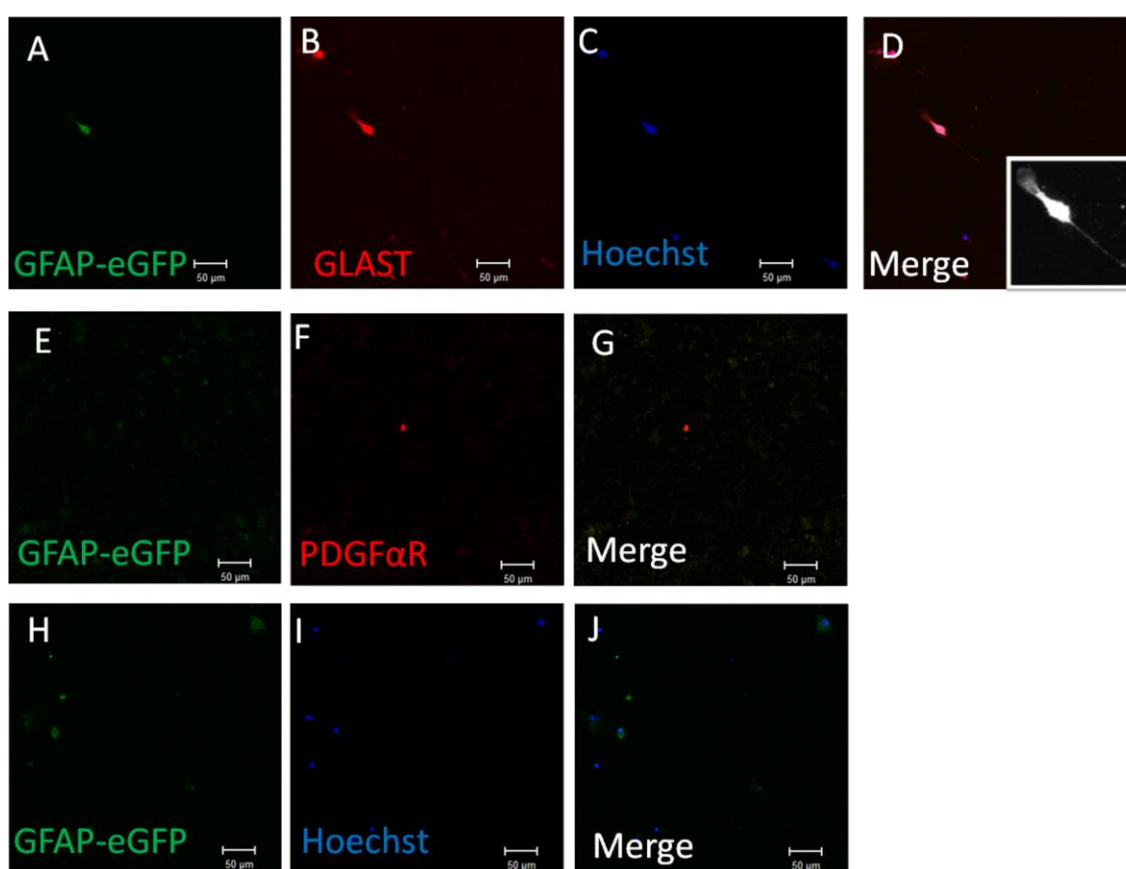

Figure13: Dissociated glial cells from cerebellum of transgenic GFAP-eGFP adult mouse. (A-D) Expression of GFP under control of GFAP promotor and expression of GLAST marker. (E-G) Expression of GFP under control of GFAP promotor and PDGFαR. (H-I) Control. Cells were cultured 7 days *in vitro* in serum media. Scale bar =50 µm, 20x

4.2 ISOLATED GLIAL CELLS

4.2.1 Isolated cells of young brain

Using the NTDK dissociation method, approximately 1.4 x 10^7 cells/brain was dissociated from pups, aged between P3 and P7, with viability of about 85 %. The dissociated young mouse brain tissue contained approximately 6 % $O4^+$ cells (cells in the column). For culturing we used the OPC-A media by Dincman et al. (2012) in order to gain the same culturing conditions for isolated glial cells from young postnatal brain. The experiments on young brain served as a positive control and the isolation of glial cells proved to work very well on the young, developing mouse brain. The cells turned out to be almost entirely $O4^+$, demonstrating the population of oligodendrocytes (Fig. 14).

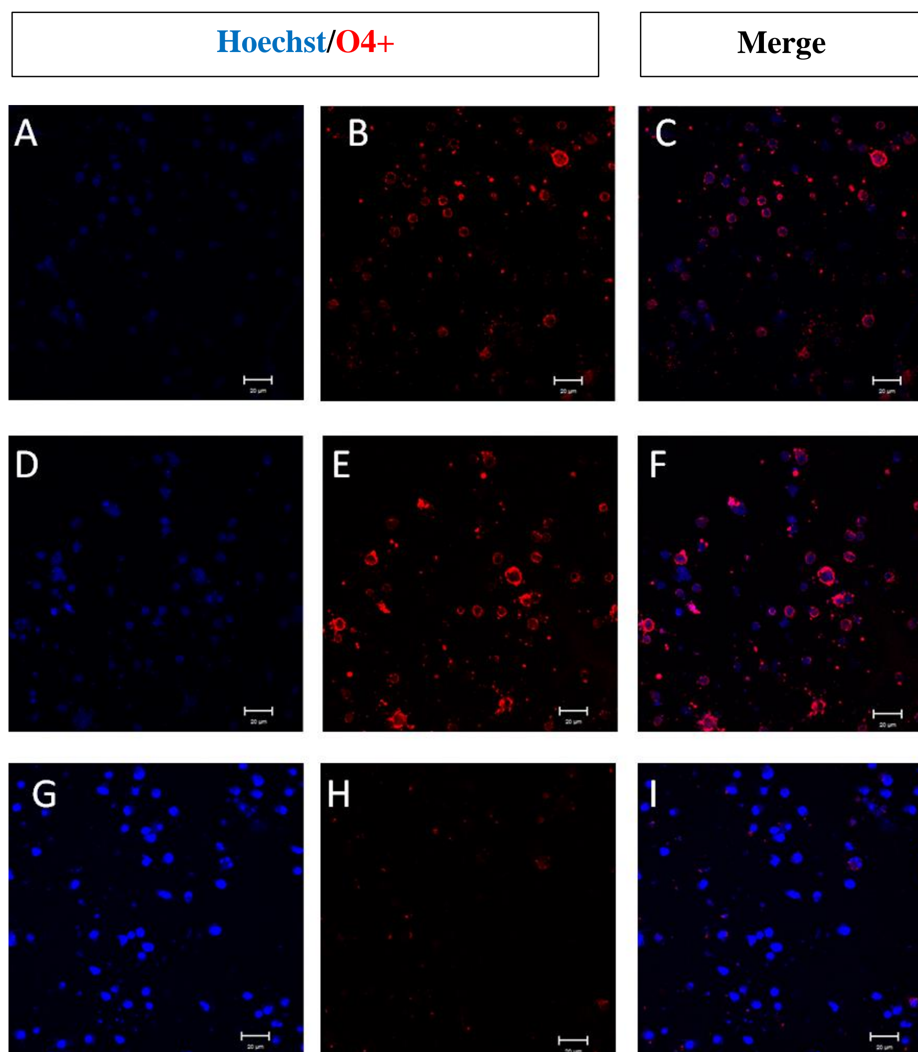

Figure 14: Isolated cells from young mouse brain. (A-F) Isolated $O4^+$ cells from P6 mouse, beforehand cultured 5 days *in vitro* in OPC-A media. (G-H) Control; scale bar = 20 µm, 20 x

4.2.2 Isolated cells of adult forebrain

The isolation of $O4^+$ cells from adult forebrain tissue with MACS cells isolation system turned out as not practicable. Approximately 54 % of the cells that were put through the column remained in the column. The isolation of adult glia remains difficult to perform and the right purification step is needed for further optimisation.

5 DISCUSSION

As glia were found to be extremely important for CNS to function, they grew into a significant part of research in the field of neuroscience. Glial cells became an important subject in cell culture studies (Megale de Almeida-Leite and Esteves Arantes, 2010), with the aim to find a way for regeneration of damaged CNS and to study the involvement of glia in numerous diseases (DeWitt et. al, 1997; Mena et al., 2002; Barres, 2008).

There are very few reports of purified and isolated glial cell populations from adult rodents as the preparation of adult CNS tissue has proved problematic (Saxena et al., 2012). We performed dissociation, cell culturing and cell isolation from adult mouse CNS tissue in order to draw experiments closer to adult human. We successfully managed to dissociate glial cells from the adult optic nerve, forebrain and cerebellum and maintained them *in vitro*. In addition, we were able to confirm that the optic nerve is a reliable and simple model to study glia (Bolton and Butt, 2004). With the ability to maintain cells in culture we also achieved and fulfilled the expectations of the 3Rs principles (Robinson, 2005). However, our experiments of glial isolation and purification with the MACS separation system preformed on the adult brain tissue remained difficult and unsuccessful. The problem most likely occurs because of the higher amount of myelin in adult brain tissue (Young, et al., 2013), which glues all the cells together so the MACS is not able to separate the cells marked with magnetic beads. Moreover, besides glia, the adult mammalian brain consists of a variety of projection neurons, local interneurons and has a complex nature of extracellular matrix (Saxena et al., 2012), which certainly makes the purification and isolation of glial cells even more problematic.

According to the MACS's original Anti-O4 MicroBeads protocol, the use of P3-P7 dissociated mouse brain tissue is suggested, and should contain approximately 5-10 % of O4+ cells (MACS Miltenyi Biotec, 2011a). Our control experiments on the young brain were consistent with that. As many previous reports have shown (Dincman et al., 2012; Chen et al., 2007), we were able to confirm that the isolation of glia is practicable and works well on early postnatal, developing brain.

Since we have managed to optimise the in-house dissociation method and made it scientifically useful, it obtained an advantage over NTDK dissociation method in the sense of financial aspect. As generally known, kits are usually very expensive. Thus, the in-house method can be used as an alternative dissociation method, when there is no substantial reason for the use of the kit.

The use of the two different media had no significant effect in our experiments. According to Raff et al. (1984), populations of astrocytes or oligodendrocyte can be obtained, depending on presence or absence of serum in the media. This aspect might be of greater interest if the isolation of adult O4+ cells from adults would be practicable. In that case, we could determine if the growth and cell proliferation of isolated O4+ cells in culture would be better in serum free media, which should stimulate the development of oligodendrocytes. Nevertheless, we managed to characterise oligodendroglia and astroglia dissociated from adult mouse CNS by their specific markers. We detected O4+, Sox10+ cells which refer to oligodendrocytes (Takahashi and Suzuki, 2012; Stolt et al., 2002) and on the other hand GLAST+ and GFAP+ cells, which represent astrocytes (Chaudhry et al., 1995 Gomes et al., 1999). We also characterised populations of OPCs (or O-2A) in culture, with their typical bipolar morphology (Butt et al., 2005).

6 CONCLUSION

Although the isolation of pure glia populations from adult mice brain failed in our case, there is still a possibility that this can be accomplished in the future. The first thing required, in order to achieve this goal, is to find an appropriate purification process to resolve the problem of excess myelin before cells go through the magnetic separation procedure. If the myelin issue turns out to be the main problem in accomplishing glial isolation, and we overcome it, the doors to research on adult isolated glial cell populations will be open. By carrying out a variety of methods, such as quantitative polymerase chain reaction (qPCR), Western blotting and proteomics on these adult glia, we will be able to learn more about individual diseases such as AD, MS, PD, and their connection to astrocyte and oligodendrocyte populations in adult humans, in which these diseases most likely occur. Moreover, testing the important pathways, e.g. GSK3β, related to neurodegenerative diseases and their potential therapeutics might be much more applicable on cells isolated from adult mice and could give us more accurate results.

Despite the issues we still need to overcome, we succeeded in dissociating glia from adult mouse CNS and created a dependable *in vitro* model of mixed glia populations, which still represents an advance in the study of glia in the future.

7 SUMMARY

Glial cells are the most numerous cells in mammalian CNS and have common origin with neurons from the embryonic germ layer or neuroectoderm. Glia are categorised in macroglia (astrocytes, oligodendrocytes, ependymal cells) and microglia (the immune system of the CNS). Schwann cells are the main representative of glia in PNS (Verkhratsky and Butt, 2007). *In vitro* cultures of glial cells became a useful approach to study glial characteristics and physiology in order to develop novel drugs to treat neurodegenerative disorders (Heneka et al., 2010). However, the majority of *in vitro* studies are performed on embryonic and postnatal brains, whereas most of the neurodegenerative diseases develop in adult brain. Consequently, it is important to develop models that can allow us to study adult glia *in vitro* and help us develop drugs to treat adult neurodegenerative diseases. The aim of our study was to develop a reliable technique for isolation of pure populations of oligodendrocytes and astrocytes from adult mouse CNS.

The most important functions of astrocytes are the regulation of the microenvironment, metabolic and structural support to neurons, involvement in the information-transfer system, as well as building the BBB and secreting numerous gliotransmitters, which control the efficacy of the synapse. Because of their large number in the brain, astrocytes take part in several neurodegenerative degenerative diseases such as AD (Heneka et al., 2010). The main function of oligodendrocytes is the production of myelin, which isolates axons and provides fast transmission of impulses (Barres, 2008). Oligodendrocytes develop from OPCs, which in adult brain also represent a source of new oligodendrocytes after injury (Wolswijk et al., 1990). O-2A cells were discovered in the culture of the optic nerve. These cells have the ability to develop into type 2 astrocytes or oligodendrocytes, depending on the presence or absence of serum in the culturing media (Raff et al., 1984).

Our experiments were based on dissection of the forebrain, cerebellum and optic nerve tissue of adult mouse, cell dissociation, cell isolation with MACS separation system, cell culturing and immunohistochemistry. The latter was performed with astrocyte (GFAP, GLAST) and oligodendrocyte (Sox10, O4 and PDGFαR) specific markers. For the dissociation of the mouse CNS tissue we used in-house and NTDK methods in order to

compare them. The serum and serum free media were used to see their effect on differentiation of glial cells. We also performed experiments on young postnatal brains as a positive control.

We were able to characterise populations of astrocytes, oligodendrocytes and OPCs (or O-2A) in cultures from the brain and optic nerve of adult mouse. Our results also confirmed that O4 and GLAST are appropriate markers to preform cell isolation. Both, in-house and NTDK methods, turned out to be successful dissociation methods. NTDK method was found to be applicable not only for the dissociation of brain tissue but also for the adult mouse optic nerve tissue. The dissociation of adult mouse forebrain tissue provided us with a sufficient number of cells to perform cell isolation. We used the NTDK method in order to make it compatible with the MACS system. No significant effects were observed despite using two different media in our experiments. We also proved that the process of dissociation has no negative effect on cells from transgenic reporter mice, which continued to express reporters *in vitro*. The isolation of pure populations of glia from adult mouse CNS with the MACS isolation system turned out as non-practicable.

We managed to dissociate glia from adult mouse CNS and maintained them *in vitro* as a model of mixed glia populations. However, we did not accomplish our aim of isolating pure cell populations. Because of the high amount of myelin (Young et al., 2013) and complex extracellular matrix in adult brain (Saxena et al., 2012), the isolation of pure glial populations from adult mouse CNS was not possible. We successfully optimised an in-house dissociation method, which is cheaper and thus preferable to the NTDK one, if there is no special need to use the latter.

Despite our unsuccessful attempt to gain pure isolated adult glia populations *in vitro*, there is still hope to accomplish this in the future. To reach this goal, we first need to find a solution to extracting myelin in the purification process. Once we are able to culture pure adult astrocyte and oligodendrocyte, we will be able to perform different biological experiments regarding neurodegenerative disorders. This could potentially lead to new and more successful ways of treatment of neurodegenerative diseases on adult humans.

7.1 POVZETEK

Celice glije so najštevilčnejše celice v centralnem živčnem sistemu (CŽS) sesalcev. Imajo skupen izvor z nevroni iz embrionalne zarodne plasti, nevroektoderma. Glijo delimo na makroglijo v katero uvrščamo astrocite, oligodendrocite in ependimske celice ter mikroglijo, ki predstavlja imunski sistem CŽS. Glavni predstavnik glije v perifernem živčnem sistemu (PŽS) so Schwannove celice (Verkhratsky in Butt, 2007) . *In vitro* kulture celic glija so postale uporabno orodje za proučevanje lastnosti in fiziologije glije za razvoj novih zdravil in zdravljenje nevrodegenerativnih bolezni (Heneka in sod., 2010). Vendar pa se večina študij *in vitro* izvaja na embrionalnih in postnatalnih možganih, medtem ko se največ nevrodegenerativnih bolezni pojavi v možganih odraslih. Zato je pomembno, da se razvije modele, ki nam bodo omogočili raziskave na odraslih celicah glija *in vitro* ter nam tako pomagali pri razvoju zdravil, ki bodo primerna za zdravljenje odraslih ljudi. Namen naše raziskave je bil razviti zanesljivo tehniko za izolacijo čistih populacij oligodendrocitov in astrocitov iz CŽS odrasle miši. Postavili smo hipotezo, da lahko izoliramo astrocite in oligodendrocite iz odraslega CŽS miši z uporabo magnetno pogojenega ločevanja celic (*ang*. MACS) ter izolirane celice vzdržujemo v kulturi za nadaljnje biološke eksperimente.

Astrociti se razvijejo iz prekurzorjev imenovanih radialne glija celice, ki sodelujejo pri migraciji nevronov v razvijajočih se možganih (Anton in sod., 1996; Gomes in sod., 1999). Po morfologiji razlikujemo dva tipa astrocitov. Fibrilarni (ali tip 1 *in vitro*) astrociti naseljujejo belino CŽS in imajo bolj pravilno, zvezdasto obliko, s celindričnimi izrastki (Vaughn in Pease, 1967, cit. po Molofsly in sod., 2012). Protoplazemski (ali tip 2) astrociti naseljujejo sivino in imajo bolj nepravilno obliko (Molofsly in sod., 2012). Najpomembnejše funkcije astrocitov so regulacija mikrookolja, predstavljajo metabolno in strukturno podporo nevronom, sodelujejo pri prenosu informacij, gradijo krvno-možgansko pregrado in izločajo številne gliotransmiterje, kot so glutamat, ATP, GABA, D-serin, GABA, taurin, ki nadzorujejo učinkovitost sinapse (Heneka in sod., 2010). Astrociti predstavljajo obrambni mehanizem CŽS s procesom reaktivne astroglioze, ki pa se lahko razvije v škodljiv proces in povzroči številne nevropatološke motnje, npr. Alzheimerjeva in

Parkinsonova bolezen (Nagele in sod., 2004). Astrociti izražajo specifične označevalce (GFAP, GLAST, vimentin), ki predstavljajo pomembno orodje za detekcijo in karakterizacijo astroglije (Gomes in sod., 1999; Rothstein, 1994).

Oligodendrocit so celice, ki v CŽS proizvajajo mielin. Razvijejo se iz migrirajočih in mitotičnih prekurzorskih celic oligodendrocitov (*ang.* OPCs) (Baumann in Pham-Dinh, 2001). Glede na morfologijo, velikost in debelino mielinske ovojnice ločimo štiri različne tipe oligodendrocitov. Tip I in II se pojavita pozo v razvoju medtem, ko se oligodendrociti tip III in IV pojavijo kasneje in mielinizirajo predvsem aksone večjih diametrov (Butt, 2013). Glavna funkcija mielina je izolacija aksonov, ki poskrbi za hiter prenos impulzov. Poleg električnih lastnosti s katerimi oligodendrociti oskrbujejo aksone, za slednje predstavljajo tudi strukturno podporo (Barres, 2008). Suha masa mielina vsebuje 70 % lipidov in 30 % proteinov. V mielinu najdemo številne specifične proteine, kot so mielinski bazični protein (*ang.* MBP), proteolipid protein (*ang.* PLP) in z mielinom povezan glikoprotein (*ang.* MAG) (Ndubaku in de Bellard, 2008). V zadnjem desetletju so znanstveniki odkrili nov razred glije imenovan NG2 glija. Te celice, ki so predhodno veljale za OPC, naseljujejo tako belino kot sivino CŽS. Identificirane so bile kot kompleksne postmitotične celice, ki se vključujejo v nevro-glija interakcije in formirajo glialne brazgotine, kot odgovor na poškodbe CŽS (Butt, 2005). Oligodendrogijo je možno zaznati in karakterizirati preko številnih označevalcev, specifičnih za OPC in zrele oligodendrocite (O4, Sox10, APC, Olig1, Olig2, PDGFαR, NG2). Oligodendrociti igrajo veliko vlogo tudi v nevropatologiji, saj so zaradi svoje visoke metabolne aktivnosti izpostavljeni oksidativnem stresu, ki lahko povzroči številne, predvsem z demielinizacijo povezane bolezni, npr. multipla skleroza (Bradl in Lassman, 2010).

Mikroglija izvira iz mieloidne linije, njena glavna vloga pa je imunska zaščita CŽS, saj imajo celice mikroglije značilnosti makrofagov, s sposobnostjo fagocitoze (Heneka in sod., 2010). V primeru poškodbe CŽS, mikroglija poskrbi za odstranitev poškodovanih ali odmrlih celic ter zgradi pregrado med zdravim in poškodovanim tkivom (Davalos in sod., 2005). Poleg tega mikroglija izloča številne citokine in pro-vnetne mediatorje, npr. TNF-α, ter na ta način spodbuja nastajanje novih oligodendrocitov (Arrnet in sod., 2001, cit. po Barres, 2008). Kljub temu pa je mikroglija s svojimi nevro-vnetnimi mehanizmi vpletena v

napredovanje bolezni, kot so Parkinsonova in Alzheimerjeva bolezen (Heneka in sod., 2010). Aktivirana mikroglija v prihodnosti lahko prispeva k razvoju potencialnih protivnetnih terapij (Sastre in sod., 2006).

V razvijajočih se možganih in v določenih regijah postnatalnih in odraslih možganov so prisotne tako imenovane živčne matične celice (*ang.* NMC). Te celice se razvijejo ne le v nevrone temveč tudi v celice glije (Alvarez - Buylla in Lim, 2004). Nedavne študije so pokazale, da glija celice v razvoju, radialna glija in podpopulacije astrocitov pri odraslih sesalcih, delujejo kot NMC (Kriegstein in Alvarez-Buylla, 2009). Te matične celice so lahko pomemben rezervoar prekurzorskih celic in proizvedejo širok spekter podtipov živčnih celic za popravilo možganov, kar lahko vodi do razvoja novih terapevtskih aplikacij (Lindvall in Kokaia, 2006).

Signalne mulekule Wnt so iz velike družine glikoproteinov. Njihova signalna pot regulira embrionalno vzorčenje, celično proliferacijo in determinacijo, anatomijo nevralnega citoskeleta, diferenciacijo sinaps v malih možganih in regulira apoptozo (Patapoutian in Richardt , 2000). Signalna pot Wnt je pod kontrolo β-katenina. Ob vezavi Wnt molekule na membranske receptorje ta posredno sproži inhibicijo glikogen sintazne kinaze-3β (GSK-3β), kar se izrazi v stabilizaciji in jedrni translokaciji β-katenina, ki formira jedrni kompleks z drugimi proteini in tanskripcijskimi dejavniki ter tako aktivira številne gene. V odsotnosti β-katenina, GSK-3β s pomočjo ostalih dejavnikov povzroči razgradnjo β-katenina in posledično tudi inhibicijo izražanja genov Wnt. Azim in Butt (2011) sta s številnimi inhibitorji (ARA-014418, litij, indirubin in L803-mt) inhibirala GSK-3β, kar se je izrazilo v povečani proliferaciji in preživetju OPC celic, večji diferenciaciji oligodendrocitov in mielinizaciji. Poleg tega se je izkazalo, da ima inhibicija GSK-3β vpliv tudi na regeneracijo in remielinizacijo oligodendrocitov, kar predstavlja možnost za razvoj novih zdravil za bolezni, kot je multipla skleroza. Inhibicija GSK-3β pa se je izkazala za ključno tudi pri astrogliozi. Povzročila je nastajanje novih astrocitov, ki bi lahko pomenili ključni faktor pri popravljanju poškodb in zdravljenju bolezni CŽS (Rivera, 2014).

Optični živec se je izkazal kot izvrsten model za raziskovanje številnih lastnostih glije in interakcij z aksoni, saj ga je enostavno izolirati in ne vsebuje celičnih teles nevronov

(Bolton in Butt, 2004). V celični kulturi optičnega živca so odkrili O-2A celice. Te celice imajo sposobnost, da se razvijejo v astrocite tipa 2 ali oligodendrocite, odvisno od prisotnosti ali odsotnosti seruma v gojitvenem mediju (Raff et al., 1984). Na ta način lahko manipuliramo celice in njihovo okolje, kar nam omogoča raziskovanje njihovega diferenciacijskega potenciala in karakterizacijo (Hardy in Reynolds, 1991). Večino znanja s področja celične biologije glije je bilo pridobljenega s študijami linij glije ali celic v kulturi, ki so bile izolirane iz embrionalnih ali zgodnjih postnatalnih možganov (McCarthy in de Vellis, 1980; Dincman in sod., 2012; Chen in sod., 2007; Emery in Dugas, 2013). Vendar, pa glija celice iz prenatalnih in mladih možganov izražajo nejasen odnos do zrelih celic v odraslih možganih (Cahoy in sod., 2008). Ker se je izkazalo, da je odraslo glijo zelo težko izolirati in proučevati je ključno, da razvijemo stabilen *in vitro* model s čistimi celičnimi populacijami glije, izolirane iz odraslega CNS tkiva.

Pri naših poskusih smo uporabili divji tip odraslih miši in mlade miši, stare od 3 do 7 dni. Za kontrolo smo uporabili tudi odrasle transgene GFAP-eGFP miši. Vse so bile usmrčene na human način, z dislokacijo vratnih vretenc. S seciranjem smo zbrali vzorce velikih možganov ali malih možganov ali vzorec šestih optičnih živcev. Za celično disociacijo smo testirali dve metodi-domačo in metodo z uporabo kompleta za disociacijo živčnega tkiva (*ang*. NTDK). Domača metoda temelji na encimatski disociaciji s tripsinom, ki poruši integriteto tkiv z razgradnjo adhezijskih proteinov. NTDK prav tako deluje na principu encimatske razgradnje, in sicer na bazi papaina ali tripsina, odvisno od tega ali želimo disociirati pretežno astrocite ali oligodendrocite. Princip MACS izolacijskega sistema temelji na označevanju celic s protitelesi opremljenimi z magnetnimi delci; v našem primeru označevanje $O4^+$ celic z Anti-O4 MicroBeads. Nato se celice spusti skozi kolono, ki se jo predhodno namesti v magnetno polje MACS separatorja. Celice označene z magnetnimi delci ostanejo v koloni medtem, ko ostale celice stečejo skozi. Kolono se nato odstrani iz magnetnega polja in magnetno označene celice se eluira kot pozitivno celično frakcijo. Po disociaciji in izolaciji celic smo celice nanesli na krovna stekelca z gostoto približno 10^6 celic/cm^2 in jih kultivirali 5-7 dni na 37 °C in 5 % CO_2. Po končani kultivaciji smo celice uporabili za izvedbo imunohistokemijske metode. Slike imunsko označenih celic smo na koncu pridobil s konfokalnim laserskim mikroskopom.

S celično disociacijo smo se osredotočili na odraslo tkivo optičnega živca z uporabo svoje lastne in NTDK disociacijske metode. Za kultivacijo celic smo uporabili dva različna gojitvena medija, in sicer s serumom (NCS medij) in brez seruma (NB-A medij). Disociacija velikih in malih možganov, z uporabo NTDK metode, je bila prvotno izvedena kot pozitivna kontrola. Domača metoda se je izkazala za uspešno metodo disociacije glija celic iz odraslega optičnega živca miši, saj smo celice lahko vzdrževali v kulturi 7 dni. Poleg tega smo uspeli detektirati tako astrocite kot oligodendrocite z njihovima specifičnima označevalcema, GFAP in Sox10. Celice, identificirane s Sox10, so imele karakteristike bipolarne morfologije zgodnjih OPC (ali O-2A) v kulturi. Z uporabo NTDK metode smo dokazali, da je ta primerna tudi za disociacijo tkiva optičnega živca odrasle miši glede na to, da je ta metoda prvotno namenjena za disociacijo možganskega mišjega tkiva (MACS Miltenyi Biotec, 2011). Metoda kompleta je bila prvotno uporabljena zaradi svoje večje kompatibilnosti z izolacijskim sistemom MACS. Z istim namenom smo se osredotočili tudi na testiranje označevalcev GLAST in O4, da bi preverili ali sta dobra markerja za nadaljnje poskuse izolacije z Anti-O4 MicroBeads in Anti-GLAST (ACCSA-1) MicroBead Kit. Zaradi pomanjkanja časa smo se kasneje odločili le za izolacijo $O4^+$ celic z Anti-O4 MicroBeads in nadaljevali z disocijacijskim kompletom baziranem na papainu za disocaicijo oligodendrocitov. Obe disociacijski metodi sta se izkazali za uspešni, saj je bilo celice z obema metodama mogoče vzdrževati v kulturi 7 dni. Disociacija tkiva velikih možganov odrasle miši z metodo NTDK je bila prvotno namenjena kot kontrola disociaciji tkiva optičnega živca. Vendar, pa nam je odraslo možgansko tkivo z uporabo metode NDTK doprineslo zadostno število celic za nadaljnjo izolacijo z sistemom MACS. Imuno označevanje z PDGFαR primarnimi protitelesi je bilo izraženo skupaj z GFAP, ki je močni označevalec astroglije. To predstavlja karakteristiko O-2A, ki imajo bipolarno morfologijo in so-izražajo označevalce OPC in GFAP. Dokazali smo tudi, da proces disociacije nima negativnega učinka na celice pridobljene iz transgene miši, saj celice obdržijo sposobnost izražanja reporterskega gena *in vitro*. Izolacija $O4^+$ celice iz možganov mladih miši, starih od 3 do 7 dni se je izkazala za učinkovito in nam je služila kot pozitivna kontrola. Vendar, pa izolacija $O4^+$ celic iz odraslih velikih možganov miši z uporabo sistema MACS ni uspela in se je izkazala za neizvedljivo. Od celic, ki so bile spuščene skozi kolono, jih je v povprečju kar 54 % ostalo v koloni. To pomeni, da je

za pridobitev pozitivnih rezultatov potrebno najti primeren purifikacijski postopek in na ta način optimizirati proces izolacije.

Uspelo nam je disociirati celice glija iz CŽS odrasle miši jih in ohranjati *in vitro*, kot model mešanih populacij glija. Kljub temu nismo dosegli našega cilja, saj nam ni uspelo izolirati posameznih populacij glije. Vzrok je predvsem v visoki vsebnosti mielina (Young in sod., 2013) in kompleksnega ekstracelularnega matriksa v odraslih možganih (Saxena et al., 2012), kar močno otežuje postopek izolacije in pridobitev čistih celičnih populacij. Uspešno smo optimizirali lastno disociacijsko metodo. Slednja ima v ekonomskem smislu prednost pred metodo NTDK, saj je cenejša in tako bolj zaželena za uporabo, kadar ni posebne potrebe za uporabo kompleta.

Kljub našemu neuspelemu poskusu izolacije odraslih populacij glija *in vitro*, je še vedno možnost, da to dosežemo v prihodnje. Za dosega tega cilja moramo najprej najti rešitev za ekstrakcijo mielina pri procesu purifikacije. Kultivacija čistih odraslih astrocitov in oligodendrocitov bi nam omogočila izvajanje številnih bioloških ekperimentov v povezavi z nevrodegenerativnimi boleznimi. To pa bi lahko potencialno vodilo k odkrivanju novih in bolj uspešnih zdravljenj teh bolezni pri odraslem človeku.

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